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(54) Title: RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE

(57) Abstract: The present invention relates to a Drosophila in vitro system which was used to demonstrate that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length. Furthermore, when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of long dsRNA. Thus , these 21-23 nt fragments are the sequence-specific mediators of RNA degradation. A molecular signal, which may be their specific length, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells, where the use of long dsRNAs to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. This specific targeting of a particular gene function is useful in functional genomic and therapeutic applications.

RNA Sequence-Specific Mediators of RNA Interference

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/265,232, filed January 31, 2001 and U.S. Provisional Application No. 60/193,594, filed March 30, 2000, and claims priority under 35 U.S.C. §119 to European Application No. 00 126 325.0 filed December 1, 2000. The entire teachings of the above applications are incorporated herein by reference.

GOVERNMENT SUPPORT

Work described herein was funded in part by grants from the National Institutes of Health through a United States Public Health Service MERIT award (Grant No. RO1-GM34277) from the National Institutes of Health. The United States government has certain rights in the invention.

BACKGROUND OF THE INVENTION

RNA interference or "RNAi" is a term initially coined by Fire and co-workers to describe the observation that double-stranded RNA (dsRNA) can block gene expression when it is introduced into worms (Fire et al. (1998) Nature 391, 806-811). dsRNA directs gene-specific, post-transcriptional silencing in many organisms, including vertebrates, and has provided a new tool for studying gene function. RNAi involves mRNA degradation, but many of the biochemical mechanisms underlying this interference are unknown. The recapitulation of the essential features of RNAi in vitro is needed for a biochemical analysis of the phenomenon.

SUMMARY OF THE INVENTION

Described herein is gene-specific, dsRNA-mediated interference in a cell-free system derived from syncytial blastoderm Drosophila embryos. The in vitro system complements genetic approaches to dissecting the molecular basis of

RNAi. As described herein, the molecular mechanisms underlying RNAi were examined using the Drosophila in vitro system. Results showed that RNAi is ATP-dependent yet uncoupled from mRNA translation. That is, protein synthesis is not required for RNAi in vitro. In the RNAi reaction, both strands (sense and antisense) of the dsRNA are processed to small RNA fragments or segments of from about 21 to about 23 nucleotides (nt) in length (RNAs with mobility in sequencing gels that correspond to markers that are 21-23 nt in length, optionally referred to as 21-23 nt RNA). Processing of the dsRNA to the small RNA fragments does not require the targeted mRNA, which demonstrates that the small RNA species is generated by processing of the dsRNA and not as a product of dsRNA-targeted mRNA degradation. The mRNA is cleaved only within the region of identity with the dsRNA. Cleavage occurs at sites 21-23 nucleotides apart, the same interval observed for the dsRNA itself, suggesting that the 21-23 nucleotide fragments from the dsRNA are guiding mRNA cleavage. That purified 21-23 nt RNAs mediate RNAi confirms that these fragments are guiding mRNA cleavage.

Accordingly, the present invention relates to isolated RNA molecules (double- stranded; single-stranded) of from about 21 to about 23 nucleotides which mediate RNAi. That is, the isolated RNAs of the present invention mediate degradation of mRNA of a gene to which the mRNA corresponds (mediate degradation of mRNA that is the transcriptional product of the gene, which is also referred to as a target gene). For convenience, such mRNA is also referred to herein as mRNA to be degraded. As used herein, the terms RNA, RNA molecule(s), RNA segment(s) and RNA fragment(s) are used interchangeably to refer to RNA that mediates RNA interference. These terms include double-stranded RNA, single-stranded RNA, isolated RNA (partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA), as well as altered RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of the 21-23 nt RNA or internally (at one or more nucleotides of the RNA). Nucleotides in the RNA molecules of the present invention can also comprise non-standard nucleotides, including non-naturally occurring nucleotides or deoxyribonucleotides. Collectively, all such altered RNAs are referred to as analogs or analogs of naturally-occurring RNA. RNA of 21-23 nucleotides of the present invention need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNAi. As used herein the phrase "mediates RNAi" refers to (indicates) the ability to distinguish which RNAs are to be degraded by the RNAi machinery or process. RNA that mediates RNAi interacts with the RNAi machinery such that it directs the machinery to degrade particular mRNAs. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which their sequence corresponds. It is not necessary that there be perfect correspondence of the sequences, but the correspondence must be sufficient to enable the RNA to direct RNAi cleavage of the target mRNA. In a particular embodiment, the 21-23 nt RNA molecules of the present invention comprise a 3' hydroxyl group.

The present invention also relates to methods of producing RNA molecules of about 21 to about 23 nucleotides with the ability to mediate RNAi cleavage. In one embodiment, the Drosophila in vitro system is used. In this embodiment, dsRNA is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA molecules of about 21 to about 23 nucleotides. In another embodiment, the Drosophila in vitro system is used to obtain RNA sequences of about 21 to about 23 nucleotides which mediate RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene to be targeted is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides in length. As shown herein, 21- 23 nt RNA mediates RNAi of the mRNA of the targeted gene (the gene whose mRNA is to be degraded). The method of obtaining 21-23 nt RNAs using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination.

The present invention also relates to 21-23 nt RNA produced by the methods of the present invention, as well as to 21-23 nt RNAs, produced by other methods, such as chemical synthesis or recombinant DNA techniques, that have the same or substantially the same sequences as naturally-occurring RNAs that mediate RNAi, such as those produced by the methods of the present invention. All of these are referred to as 21-23 nt RNAs that mediate RNA interference. As used herein, the term isolated RNA includes RNA obtained by any means, including processing or cleavage of dsRNA as described herein; production by chemical synthetic methods; and production by recombinant DNA techniques. The invention further relates to uses of the 21-23 nt RNAs, such as for therapeutic or prophylactic treatment and compositions comprising 21-23 nt RNAs that mediate RNAi, such as pharmaceutical compositions comprising 21-23 nt RNAs and an appropriate carrier (e.g., a buffer or water).

The present invention also relates to a method of mediating RNA interference of mRNA of a gene in a cell or organism (e.g., mammal such as a mouse or a human). In one embodiment, RNA of about 21 to about 23 nt which targets the mRNA to be degraded is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. The cell or organism can be one in which RNAi occurs as the cell or organism is obtained or a cell or organism can be one that has been modified so that RNAi occurs (e.g., by addition of components obtained from a cell or cell extract that mediate RNAi or activation of endogenous components). As used herein, the term "cell or organism in which RNAi occurs" includes both a cell or organism in which RNAi occurs as the cell or organism is obtained, or a cell or organism that has been modified so that RNAi occurs. In another embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNAs of about 21 to about 23 nucleotides. 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. As described for the previous embodiment, the cell or organism is one in which RNAi occurs naturally (in the cell or organism as obtained) or has been modified in such a manner that RNAi occurs. 21 to 23 nt RNAs can also be produced by other methods, such as chemical synthetic methods or recombinant DNA techniques.

The present invention also relates to biochemical components of a cell, such as a Drosophila cell, that process dsRNA to RNA of about 21 to about 23 nucleotides. In addition, biochemical components of a cell that are involved in targeting of mRNA by RNA of about 21 to about 23 nucleotides are the subject of the present invention. In both embodiments, the biochemical components can be obtained from a cell in which they occur or can be produced by other methods, such as chemical synthesis or recombinant DNA methods. As used herein, the term "isolated" includes materials (e.g., biochemical components, RNA) obtained from a source in which they occur and materials produced by methods such as chemical synthesis or recombinant nucleic acid (DNA, RNA) methods.

The present invention also relates to a method for knocking down (partially or completely) the targeted gene, thus providing an alternative to presently available methods of knocking down (or out) a gene or genes. This method of knocking down gene expression can be used therapeutically or for research (e.g., to generate models of disease states, to examine the function of a gene, to assess whether an agent acts on a gene, to validate targets for drug discovery). In those instances in which gene function is eliminated, the resulting cell or organism can also be referred to as a knockout. One embodiment of the method of producing knockdown cells and organisms comprises introducing into a cell or organism in which a gene (referred to as a targeted gene) is to be knocked down, RNA of about 21 to about 23 nt that targets the gene and maintaining the resulting cell or organism under conditions under which RNAi occurs, resulting in degradation of the mRNA of the targeted gene, thereby producing knockdown cells or organisms. Knockdown cells and organisms produced by the present method are also the subject of this invention.

The present invention also relates to a method of examining or assessing the function of a gene in a cell or organism. In one embodiment, RNA of about 21 to about 23 nt which targets mRNA of the gene for degradation is introduced into a cell or organism in which RNAi occurs. The cell or organism is referred to as a test cell or organism. The test cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control cell or organism, such as a corresponding cell or organism that is treated in the same manner except that the targeted (specific) gene is not targeted. A 21 to 23 nt RNA that does not target the mRNA for degradation can be introduced into the control cell or organism in place of the RNA introduced into the test cell or organism, although it is not necessary to do so. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the degraded mRNA. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract that mediates RNAi, such as the soluble extract derived from Drosophila embryo described herein, under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into a cell or organism in which RNAi occurs (test cell or test organism). The test cell or test organism is maintained under conditions under which degradation of the mRNA occurs. The phenotype of the test cell or organism is then observed and compared to that of an appropriate control, such as a corresponding cell or organism that is treated in the same manner as the test cell or organism except that the targeted gene is not targeted. A difference between the phenotypes of the test and control cells or organisms provides information about the function of the targeted gene. The information provided may be sufficient to identify (define) the function of the gene or may be used in conjunction with information obtained from other assays or analyses to do so.

Also the subject of the present invention is a method of validating whether an agent acts on a gene. In this method, RNA of from about 21 to about 23 nucleotides that targets the mRNA to be degraded is introduced into a cell or organism in which RNAi occurs. The cell or organism (which contains the introduced RNA) is maintained under conditions under which degradation of mRNA occurs, and the agent is introduced into the cell or organism. Whether the agent has an effect on the cell or organism is determined; if the agent has no effect on the cell or organism, then the agent acts on the gene.

The present invention also relates to a method of validating whether a gene product is a target for drug discovery or development. RNA of from about 21 to about 23 nucleotides that targets the mRNA that corresponds to the gene for degradation is introduced into a cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA occurs, resulting in decreased expression of the gene. Whether decreased expression of the gene has an effect on the cell or organism is determined, wherein if decreased expression of the gene has an effect, then the gene product is a target for drug discovery or development.

The present invention also encompasses a method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides which targets the mRNA of the protein (the mRNA that encodes the protein) for degradation. As a result, the protein is not produced or is not produced to the extent it would be in the absence of the treatment.

Also encompassed by the present invention is a gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

Also encompassed by the present invention is a method of identifying target sites within an mRNA that are particularly suitable for RNAi as well as a method of assessing the ability of 21-23 nt RNAs to mediate RNAi.

BRIEF DESCRIPTION OF THE DRAWINGS

The file of this patent contains at least one drawing executed in color. Copies of this patent with color drawing(s) will be provided by the Patent and Trademark Office upon request and payment of the necessary fee.

Figure 1 is a schematic representation of reporter mRNAs and dsRNAs Rr-Luc and Pp-Luc. Lengths and positions of the ssRNA, asRNA, and dsRNAs are shown as black bars relative to the Rr-Luc and Pp-Luc reporter mRNA sequences. Black rectangles indicate the two unrelated luciferase coding sequences, lines correspond to the 5' and 3' untranslated regions of the mRNAs.

Figure 2A is a graph of the ratio of luciferase activities after targeting 50 pM Pp- Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 505 bp segment of the Pp- Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of seven trials ± standard deviation. Four independently prepared lysates were used. Luciferase activity was normalized to the buffer control; a ratio equal to one indicates no gene-specific interference.

Figure 2B is a graph of the ratio of luciferase activities after targeting 50 pM Rr- Luc mRNA with 10 nM ssRNA, asRNA, or dsRNA from the 501 bp segment of the Rr- Luc gene showing gene-specific interference by dsRNA in vitro. The data are the average values of six trials ± standard deviation. A Rr-Luc/Pp-Luc ratio equal to one indicates no gene-specific interference.

Figure 3A is a schematic representation of the experimental strategy used to show that incubation in the Drosophila embryo lysate potentiates dsRNA for gene-specific interference. The same dsRNAs used in Figure 2 (or buffer) was serially preincubated using two-fold dilutions in six successive reactions with Drosophila embryo lysate, then tested for its capacity to block mRNA expression. As a control, the same amount of dsRNA (10 nM) or buffer was diluted directly in buffer and incubated with Pp-Luc and Rr-Luc mRNAs and lysate.

Figure 3B is a graph of potentiation when targeting Pp-Luc mRNA. Black columns indicate the dsRNA or the buffer was serially preincubated; white columns correspond to a direct 32-fold dilution of the dsRNA. Values were normalized to those of the buffer controls.

Figure 3C is a graph of potentiation when targeting Rr-Luc mRNA. The corresponding buffer control is shown in Figure 3B.

Figure 4 is a graph showing effect of competitor dsRNA on gene-specific interference. Increasing concentrations of nanos dsRNA (508 bp) were added to reactions containing 5 nM dsRNA (the same dsRNAs used in Figures 2A and 2B) targeting Pp-Luc mRNA (black columns, left axis) or Rr-Luc mRNA (white columns, right axis). Each reaction contained both a target mRNA (Pp-Luc for the black columns, Rr-Luc for the white) and an unrelated control mRNA (Rr-Luc for the black columns, Pp-Luc for the white). Values were normalized to the buffer control (not shown). The reactions were incubated under standard conditions (see Methods).

Figure 5A is a graph showing the effect of dsRNA on mRNA stability. Circles, Pp-Luc mRNA; squares, Rr-Luc mRNA; filled symbols, buffer incubation; open symbols, incubation with Pp-dsRNA.

Figure 5B is a graph showing the stability of Rr-Luc mRNA incubated with Rr- dsRNA or Pp-dsRNA. Filled squares, buffer; open squares, Pp-dsRNA (10 nM); open circles, Rr-dsRNA (10 nM).

Figure 5C is a graph showing the dependence on dsRNA length. The stability of the Pp-Luc mRNA was assessed after incubation in lysate in the presence of buffer or dsRNAs of different lengths. Filled squares, buffer; open circles, 49 bp dsRNA (10 nM); open inverted triangles, 149 bp dsRNA (10 nM); open triangles, 505 bp dsRNA (10 nM); open diamonds, 997 bp dsRNA (10 nM). Reactions were incubated under standard conditions (see Methods).

Figure 6 is a graph showing that RNAi Requires ATP. Creatine kinase (CK) uses creatine phosphate (CP) to regenerate ATP. Circles, +ATP, +CP, +CK; squares, -ATP, +CP, +CK; triangles, -ATP, -CP, +CK; inverted triangles, -ATP, +CP, -CK.

Figure 7A is a graph of protein synthesis, as reflected by luciferase activity produced after incubation of Rr-luc mRNA in the in vitro RNAi reaction for 1 hour, in the presence of the protein synthesis inhibitors anisomycin, cycloheximide, or chloramphenicol, relative to a reaction without any inhibitor showing that RNAi does not require mRNA translation.

Figure 7B is a graph showing translation of 7-methyl-guanosine- and adenosine- capped Pp-luc mRNAs (circles and squares, respectively) in the RNAi reaction in the absence of dsRNA, as measured by luciferase activity produced in a one-hour incubation.

Figure 7C is a graph showing incubation in an RNAi reaction of uniformly ^{32}P- radiolabeled 7-methyl-guanosine-capped Pp-luc mRNA (circles) and adenosine-capped Pp-luc mRNA (squares), in the presence (open symbols) and absence (filled symbols) of 505 bp Pp-luc dsRNA.

Figure 8A is a graph of the of the denaturing agarose-gel analysis of Pp-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 8B is a graph of the of the denaturing agarose-gel analysis of Rr-luc mRNA incubated in a standard RNAi reaction with buffer, 505 nt Pp-asRNA, or 505 bp Pp-dsRNA for the times indicated showing that asRNA causes a small amount of RNAi in vitro.

Figure 9 is a schematic of the positions of the three dsRNAs, 'A,' 'B,' and 'C,' relative to the Rr-luc mRNA.

Figure10 indicates the cleavage sites mapped onto the first 267 nt of the Rr-luc mRNA (SEQ ID NO: 1). The blue bar below the sequence indicates the position of dsRNA 'C,' and blue circles indicate the position of cleavage sites caused by this dsRNA. The green bar denotes the position of dsRNA 'B,' and green circles, the cleavage sites. The magenta bar indicates the position of dsRNA 'A,' and magenta circles, the cleavages. An exceptional cleavage within a run of 7 uracils is marked with a red arrowhead.

Figure 11 is a proposed model for RNAi. RNAi is envisioned to begin with cleavage of the dsRNA to 21-23 nt products by a dsRNA-specific nuclease, perhaps in a multiprotein complex. These short dsRNAs might then be dissociated by an ATP- dependent helicase, possibly a component of the initial complex, to 21-23 nt asRNAs that could then target the mRNA for cleavage. The short asRNAs are imagined to remain associated with the RNAi-specific proteins (circles) that were originally bound by the full-length dsRNA, thus explaining the inefficiency of asRNA to trigger RNAi in vivo and in vitro. Finally, a nuclease (triangles) would cleave the mRNA.

Figure 12 is a bar graph showing sequence-specific gene silencing by 21-23 nt fragments. Ratio of luciferase activity after targeting of Pp-Luc and Rr-Luc mRNA by 5 nM Pp-Luc or Rr-Luc dsRNA (500 bp) or 21-23 nt fragments isolated from a previous incubation of the respective dsRNA in Drosophila lysate. The amount of isolated 21-23 mers present in the incubation reaction correspond to approximately the same amount of 21-23 mers generated during an incubation reaction with 5 nM 500 bp dsRNA. The data are average values of 3 trials and the standard deviation is given by error bars. Luciferase activity was normalized to the buffer control.

Figure 13A illustrates the purification of RNA fragments on a Superdex HR 200 10/30 gel filtration column (Pharmacia) using the method described in Example 4. dsRNA was 32P-labeled, and the radioactivity recovered in each column fraction is graphed. The fractions were also analyzed by denaturing gel electrophoresis (inset).

Figure 13B demonstrates the ability of the Rr-luciferase RNA, after incubation in the Drosophila lysate and fractionation as in Fig. 13A, to mediate sequence-specific interference with the expression of a Rr-luciferase target mRNA. One microliter of each resuspended fraction was tested in a 10 microliter in vitro RNAi reaction (see Example 1). This procedure yields a concentration of RNA in the standard in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. Relative luminescence per second has been normalized to the average value of the two buffer controls.

Figure 13C is the specificity control for Fig 13B. It demonstrates that the fractionated RNA of Fig 13B does not efficiently mediate sequence-specific interference with the expression of a Pp-luciferase mRNA. Assays are as in Fig 13B.

Figures 14A and 14B are schematic representations of reporter constructs and siRNA duplexes. Figure 14A illustrates the firefly (*Pp*-luc) and sea pansy (*Rr*-

luc) luciferase reporter gene regions from plasmids pGL2-Control, pGL3-Control, and pRL-TK (Promega). SV40 regulatory elements, the HSV thymidine kinase promoter, and two introns (lines) are indicated. The sequence of GL3 luciferase is 95% identical to GL2, but RL is completely unrelated to both. Luciferase expression from pGL2 is approximately 10-fold lower than from pGL3 in transfected mammalian cells. The region targeted by the siRNA duplexes is indicated as black bar below the coding region of the luciferase genes. Figure 14B shows the sense (top) and antisense (bottom) sequences of the siRNA duplexes targeting GL2 (SEQ ID Nos: 10 and 11), GL3 (SEQ ID Nos: 12 and 13), and RL (SEQ ID Nos: 14 and 15) luciferase are shown. The GL2 and GL3 siRNA duplexes differ by only 3 single nucleotide substitutions (boxed in gray). As unspecific control, a duplex with the inverted GL2 sequence, invGL2 (SEQ ID Nos: 16 and 17), was synthesized. The 2 nt 3' overhang of 2'-deoxythymidine is indicated as TT; uGL2 (SEQ ID Nos: 18 and 19) is similar to GL2 siRNA but contains ribo-uridine 3' overhangs.

Figures 15A-15J are graphs showing RNA interference by siRNA duplexes. Ratios of target to control luciferase were normalized to a buffer control (bu, black bars); gray bars indicate ratios of *Photinus pyralis* (*Pp*-luc) GL2 or GL3 luciferase to *Renilla reniformis* (*Rr*-luc) RL luciferase (left axis), white bars indicate RL to GL2 or GL3 ratios (right axis). Figures 15A, 15C, 15E, 15G, and 15I show results of experiments performed with the combination of pGL2-Control and pRL-TK reporter plasmids, Figures 15B, 15D, 15F, 15H, and 15J with pGL3-Control and pRL-TK reporter plasmids. The cell line used for the interference experiment is indicated at the top of each plot. The ratios of *Pp*-luc/*Rr*-luc for the buffer control (bu) varied between 0.5 and 10 for pGL2/pRL, and between 0.03 and 1 for pGL3/pRL, respectively, before normalization and between the various cell lines tested. The plotted data were averaged from three independent experiments ± S.D.

Figures 16A-16F are graphs showing the effects of 21 nt siRNAs, 50 bp, and 500 bp dsRNAs on luciferase expression in HeLa cells. The exact length of the long dsRNAs is indicated below the bars. Figures 16A, 16C, and 16E describe experiments performed with pGL2-Control and pRL-TK reporter plasmids, Figures 16B, 16D, and 16F with pGL3-Control and pRL-TK reporter plasmids. The data were averaged from two independent experiments ± S.D. Figures 16A, 16B, Absolute *Pp*-luc expression, plotted in arbitrary luminescence units. Figure 16C, 16D, Rr-luc expression, plotted in arbitrary luminescence units. Figures 16E, 16F, Ratios of normalized target to control luciferase. The ratios of luciferase activity for siRNA duplexes were normalized to a buffer control (bu, black bars); the luminescence ratios for 50 or 500 bp dsRNAs were normalized to the respective ratios observed for 50 and 500 bp dsRNA from humanized GFP (hG, black bars). It should be noted, that the overall differences in sequence between the 49 and 484 bp dsRNAs targeting GL2 and GL3 are not sufficient to confer specificity between GL2 and GL3 targets (43 nt uninterrupted identity in 49 bp segment, 239 nt longest uninterrupted identity in 484 bp segment) (Parrish, S., *et al., Mol. Cell, 6:*1077-1087 (2000)).

DETAILED DESCRIPTION OF THE INVENTION

Double-stranded (dsRNA) directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). The process is known to occur in a wide variety of organisms, including embryos of mammals and other vertebrates. Using the Drosophila in vitro system described herein, it has been demonstrated that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length, and furthermore, that when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of longer dsRNA. Thus, these 21-23 nt fragments are sequence-specific mediators of RNA degradation. A molecular signal, which may be the specific length of the fragments, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or recombinantly produced or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells. Use of long dsRNAs in mammalian cells to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. Specific targeting of a particular gene function, which is possible with

21-23 nt fragments of the present invention, is useful in functional genomic and therapeutic applications.

In particular, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that mediate RNAi. In one embodiment, the present invention relates to RNA molecules of about 21 to about 23 nucleotides that direct cleavage of specific mRNA to which they correspond. The 21-23 nt RNA molecules of the present invention can also comprise a 3' hydroxyl group. The 21-23 nt RNA molecules can be single-stranded or double stranded (as two 21-23 nt RNAs); such molecules can be blunt ended or comprise overhanging ends (*e.g.*, 5', 3'). In specific embodiments, the RNA molecule is double stranded and either blunt ended or comprises overhanging ends (as two 21-23 nt RNAs).

In one embodiment, at least one strand of the RNA molecule has a 3' overhang from about 1 to about 6 nucleotides (*e.g.*, pyrimidine nucleotides, purine nucleotides) in length. In other embodiments, the 3' overhang is from about 1 to about 5 nucleotides, from about 1 to about 3 nucleotides and from about 2 to about 4 nucleotides in length. In one embodiment the RNA molecule is double stranded, one strand has a 3' overhang and the other strand can be blunt-ended or have an overhang. In the embodiment in which the RNA molecule is double stranded and both strands comprise an overhang, the length of the overhangs may be the same or different for each strand. In a particular embodiment, the RNA of the present invention comprises 21 nucleotide strands which are paired and which have overhangs of from about 1 to about 3, particularly about 2, nucleotides on both 3' ends of the RNA. In order to further enhance the stability of the RNA of the present invention, the 3' overhangs can be stabilized against degradation. In one embodiment, the RNA is stabilized by including purine nucleotides, such as adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, *e.g.*, substitution of uridine 2 nucleotide 3' overhangs by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNAi. The absence of a 2' hydroxyl significantly enhances the nuclease resistance of the overhang in tissue culture medium.

The 21-23 nt RNA molecules of the present invention can be obtained using a number of techniques known to those of skill in the art. For example, the RNA can be chemically synthesized or recombinantly produced using methods known in the art. The 21-23 nt RNAs can also be obtained using the Drosophila in vitro system described herein. Use of the Drosophila in vitro system entails combining dsRNA with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the dsRNA is processed to RNA of about 21 to about 23 nucleotides. The Drosophila in vitro system can also be used to obtain RNA of about 21 to about 23 nucleotides in length which mediates RNA interference of the mRNA of a particular gene (e.g., oncogene, viral gene). In this embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double- stranded RNA is processed to the RNA of about 21 to about 23 nucleotides. As shown herein, 21-23 nt RNA mediates RNAi of the mRNA to be degraded. The present invention also relates to the 21-23 nt RNA molecules produced by the methods described herein.

In one embodiment, the methods described herein are used to identify or obtain 21-23 nt RNA molecules that are useful as sequence-specific mediators of RNA degradation and, thus, for inhibiting mRNAs, such as human mRNAs, that encode products associated with or causative of a disease or an undesirable condition. For example, production of an oncoprotein or viral protein can be inhibited in humans in order to prevent the disease or condition from occurring, limit the extent to which it occurs or reverse it. If the sequence of the gene to be targeted in humans is known, 21-23 nt RNAs can be produced and tested for their ability to mediate RNAi in a cell, such as a human or other primate cell. Those 21-23 nt human RNA molecules shown to mediate RNAi can be tested, if desired, in an appropriate animal model to further assess their in vivo effectiveness. Additional copies of 21-23 nt RNAs shown to mediate RNAi can be produced by the methods described herein.

The method of obtaining the 21-23 nt RNA sequence using the Drosophila in vitro system can further comprise isolating the RNA sequence from the combination. The 21-23 nt RNA molecules can be isolated using a number of techniques known to those of skill in the art. For example, gel electrophoresis can be used to separate 21-23 nt RNAs from the combination, gel slices comprising the RNA sequences removed and RNAs eluted from the gel slices. Alternatively, non-denaturing methods, such as non-denaturing column chromatography, can be used to isolate the RNA produced. In addition, chromatography (e.g., size exclusion chromatography), glycerol gradient centrifugation, affinity purification with antibody can be used to isolate 21-23 nt RNAs. The RNA-protein complex isolated from the Drosophila in vitro system can also be used directly in the methods described herein (e.g., method of mediating RNAi of mRNA of a gene). Soluble extracts derived from Drosophila embryo that mediate or RNAi are encompassed by the invention. The soluble Drosophila extract can be obtained in a variety of ways. For example, the soluble extract can be obtained from syncytial blastoderm Drosophila embryos as described in Examples 1, 2, and 3. Soluble extracts can be derived from other cells in which RNAi occurs. Alternatively, soluble extracts can be obtained from a cell that does not carry out RNAi. In this instance, the factors needed to mediate RNAi can be introduced into such a cell and the soluble extract is then obtained. The components of the extract can also be chemically synthesized and/or combined using methods known in the art.

Any dsRNA can be used in the methods of the present invention, provided that it has sufficient homology to the targeted gene to mediate RNAi. The sequence of the dsRNA for use in the methods of the present invention need not be known. Alternatively, the dsRNA for use in the present invention can correspond to a known sequence, such as that of an entire gene (one or more) or portion thereof. There is no upper limit on the length of the dsRNA that can be used. For example, the dsRNA can range from about 21 base pairs (bp) of the gene to the full length of the gene or more. In one embodiment, the dsRNA used in the methods of the present invention is about 1000 bp in length. In another embodiment, the dsRNA is about 500 bp in length. In yet another embodiment, the dsRNA is about 22 bp in length.

The 21 to 23 nt RNAs described herein can be used in a variety of ways. For example, the 21 to 23 nt RNA molecules can be used to mediate RNA interference of mRNA of a gene in a cell or organism. In a specific embodiment, the 21 to 23 nt RNA is introduced into human cells or a human in order to mediate RNA interference in the cells or in cells in the individual, such as to prevent or treat a disease or undesirable condition. In this method, a gene (or genes) that cause or contribute to the disease or undesirable condition is targeted and the corresponding mRNA (the transcriptional product of the targeted gene) is degraded by RNAi. In this embodiment, an RNA of about 21 to about 23 nucleotides that targets the corresponding mRNA (the mRNA of the targeted gene) for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of the corresponding mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism. In a particular embodiment, the method of mediating RNA interference of a gene in a cell comprises combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract derived from Drosophila embryo, thereby producing a combination. The combination is maintained under conditions in which the double-stranded RNA is processed to RNA of about 21 to about 23 nucleotides. The 21 to 23 nt RNA is then isolated and introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the gene in the cell or organism. In the event that the 21-23nt RNA is introduced into a cell in which RNAi, does not normally occur, the factors needed to mediate RNAi are introduced into such a cell or the expression of the needed factors is induced in such a cell. Alternatively, 21 to 23 nt RNA produced by other methods (e.g., chemical synthesis, recombinant DNA production) to have a composition the same as or sufficiently similar to a 21 to 23 nt RNA known to mediate RNAi can be similarly used to mediate RNAi. Such 21 to 23 nt RNAs can be altered by addition, deletion, substitution or modification of one or more nucleotides and/or can comprise non-nucleotide materials. A further embodiment of this invention is an ex vivo method of treating cells from an individual to degrade a gene(s) that causes or is associated with a disease or undesirable condition, such as leukemia or AIDS. In this embodiment, cells to be treated are obtained from the individual using known methods (e.g., phlebotomy or collection of bone marrow) and 21-23 nt RNAs that mediate degradation of the corresponding mRNA(s) are introduced into the cells, which are then re-introduced into the individual. If necessary, biochemical components needed for RNAi to occur can also be introduced into the cells.

The mRNA of any gene can be targeted for degradation using the methods of mediating interference of mRNA described herein. For example, any cellular or viral mRNA, can be targeted, and, as a result, the encoded protein (e.g., an oncoprotein, a viral protein), expression will be diminished. In addition, the mRNA of any protein associated with/causative of a disease or undesirable condition can be targeted for degradation using the methods described herein.

The present invention also relates to a method of examining the function of a gene in a cell or organism. In one embodiment, an RNA sequence of about 21 to about 23 nucleotides that targets mRNA of the gene for degradation is introduced into the cell or organism. The cell or organism is maintained under conditions under which degradation of mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby providing information about the function of the gene. In another embodiment, double-stranded RNA that corresponds to a sequence of the gene is combined with a soluble extract derived from Drosophila embryo under conditions in which the double-stranded RNA is processed to generate RNA of about 21 to about 23 nucleotides. The RNA of about 21 to about 23 nucleotides is isolated and then introduced into the cell or organism. The cell or organism is maintained under conditions in which degradation of the mRNA of the gene occurs. The phenotype of the cell or organism is then observed and compared to an appropriate control, thereby identifying the function of the gene.

A further aspect of this invention is a method of assessing the ability of 21-23 nt RNAs to mediate RNAi and, particularly, determining which 21-23 nt RNA(s) most efficiently mediate RNAi. In one embodiment of the method, dsRNA corresponding to a sequence of an mRNA to be degraded is combined with detectably labeled (e.g., end-labeled, such as radiolabeled) mRNA and the soluble extract of this invention, thereby producing a combination. The combination is maintained under conditions under which the double-stranded RNA is processed and the mRNA is degraded. The sites of the most effective cleavage are mapped by comparing the migration of the labeled mRNA cleavage products to markers of known length. 21 mers spanning these sites are then designed and tested for their efficiency in mediating RNAi.

Alternatively, the extract of the present invention can be used to determine whether there is a particular segment or particular segments of the mRNA corresponding to a gene which are more efficiently targeted by RNAi than other regions and, thus, can be especially useful target sites. In one embodiment, dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA of the gene is combined with a soluble extract that mediates RNAi, thereby producing a combination. The resulting combination is maintained under conditions under which the dsRNA is degraded and the sites on the mRNA that are most efficiently cleaved are identified, using known methods, such as comparison to known size standards on a sequencing gel.

OVERVIEW OF EXAMPLES

Biochemical analysis of RNAi has become possible with the development of the in vitro Drosophila embryo lysate that recapitulates dsRNA-dependent silencing of gene expression described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)). In the in vitro system, dsRNA, but not sense or asRNA, targets a corresponding mRNA for degradation, yet does not affect the stability of an unrelated control mRNA. Furthermore, pre-incubation of the dsRNA in the lysate potentiates its activity for target mRNA degradation, suggesting that the dsRNA must be converted to an active form by binding proteins in the extract or by covalent modification (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

The development of a cell-free system from syncytial blastoderm Drosophila embryos that recapitulates many of the features of RNAi is described herein. The interference observed in this reaction is sequence-specific, is promoted by dsRNA, but not by single-stranded RNA, functions by specific mRNA degradation, requires a minimum length of dsRNA and is most efficient with long dsRNA. Furthermore, preincubation of dsRNA potentiates its activity. These results demonstrate that RNAi is mediated by sequence specific processes in soluble reactions.

As described in Example 2, the in vitro system was used to analyze the requirements of RNAi and to determine the fate of the dsRNA and the mRNA. RNAi in vitro requires ATP, but does not require either mRNA translation or recognition of the 7-methyl-guanosine cap of the targeted mRNA. The dsRNA, but not single-stranded RNA, is processed in vitro to a population of 21-23 nt species. Deamination of adenosines within the dsRNA does not appear to be required for formation of the 21-23 nt RNAs. As described herein, the mRNA is cleaved only in the region corresponding to the sequence of the dsRNA and that the mRNA is cleaved at 21-23 nt intervals, strongly indicating that the 21-23 nt fragments from the dsRNA are targeting the cleavage of the mRNA. Furthermore, as described in Examples 3 and 4, when the 21-23 nt fragments are purified and added back to the soluble extract, they mediate RNA.

The present invention is illustrated by the following examples, which are not intended to be limiting in any way.

Example 1 Targeted mRNA degradation by double-stranded RNA in vitro

Materials and Methods

RNAs

Rr-Luc mRNA consisted of the 926 nt Rr luciferase coding sequence flanked by 25 nt of 5' untranslated sequence from the pSP64 plasmid polylinker and 25 nt of 3' untranslated sequence consisting of 19 nt of pSP64 plasmid polylinker sequence followed by a 6 nt Sac I site. Pp-Luc mRNA contained the 1653 nt Pp luciferase coding sequence with a Kpn I site introduced immediately before the Pp luciferase stop codon. The Pp coding sequence was flanked by 5' untranslated sequences consisting of 21 nt of pSP64 plasmid polylinker followed by the 512 nt of the 5' untranslated region (UTR) from the Drosophila hunchback mRNA and 3' untranslated sequences consisting of the 562 nt hunchback 3' UTR followed by a 6 nt Sac I site. The hunchback 3' UTR sequences used contained six G-to-U mutations that disrupt function of the Nanos Response Elements in vivo and in vitro. Both reporter mRNAs terminated in a 25 nt poly(A) tail encoded in the transcribed plasmid. For both Rr-Luc and Pp -Luc mRNAs, the transcripts were generated by run-off transcription from plasmid templates cleaved at an Nsi I site that immediately followed the 25 nt encoded poly(A) tail. To ensure that the transcripts ended with a poly(A) tail, the Nsi I-cleaved transcription templates were resected with T4 DNA Polymerase in the presence of dNTPs. The SP6 mMessage mMachine kit (Ambion) was used for in vitro transcription. Using this kit, about 80% of the resulting transcripts are 7-methyl guanosine capped. ^{32}P-radiolabeling was accomplished by including α-^{32}P-UTP in the transcription reaction.

For Pp -Luc, ss, as, and dsRNA corresponded to positions 93 to 597 relative to the start of translation, yielding a 505 bp dsRNA. For Rr -Luc, ss, as, and dsRNA corresponded to positions 118 to 618 relative to the start of translation, yielding a 501 bp dsRNA. The Drosophila nanos competitor dsRNA corresponded to positions 122 to 629 relative to the start of translation, yielding a 508 bp dsRNA. ssRNA, asRNA, and dsRNA (diagrammed in Figure 1) were transcribed in vitro with T7 RNA polymerase from templates generated by the polymerase chain reaction. After gel purification of the T7 RNA transcripts, residual DNA template was removed by treatment with RQ1 DNase (Promega). The RNA was then extracted with phenol and chloroform, and then precipitated and dissolved in water.

RNA annealing and native gel electrophoresis.

ssRNA and asRNA (0.5 μM) in 10 mM Tris-HCl (pH 7.5) with 20 mM NaCl were heated to 95 ° C for 1 min then cooled and annealed at room temperature for 12 to 16 h. The RNAs were precipitated and resuspended in lysis buffer (below). To monitor annealing, RNAs were electrophoresed in a 2% agarose gel in TBE buffer and stained with ethidium bromide (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)).

Lysate preparation

Zero- to two-hour old embryos from Oregon R flies were collected on yeasted molasses agar at 25°C. Embryos were dechorionated for 4 to 5 min in 50% (v/v) bleach, washed with water, blotted dry, and transferred to a chilled Potter-Elvehjem tissue grinder (Kontes). Embryos were lysed at 4°C in one ml of lysis buffer (100 mM potassium acetate, 30 mM HEPES-KOH, pH 7.4, 2 mM magnesium acetate) containing 5 mM dithiothreitol (DTT) and 1 mg/ml Pefabloc SC (Boehringer-Mannheim) per gram of damp embryos. The lysate was centrifuged for 25 min at 14,500 x g at 4°C, and the supernatant flash frozen in aliquots in liquid nitrogen and stored at -80°C.

Reaction conditions

Lysate preparation and reaction conditions were derived from those described by Hussain and Leibowitz (Hussain and Leibowitz, Gene 46:13-23 (1986)). Reactions contained 50% (v/v) lysate, mRNAs (10 to 50 pM final concentration), and 10% (v/v) lysis buffer containing the ssRNA, asRNA, or dsRNA (10 nM final concentration). Each reaction also contained 10 mM creatine phosphate, 10 µg/ml creatine phosphokinase, 100 µM GTP, 100 µM UTP, 100 µM CTP, 500 µM ATP, 5 µM DTT, 0.1 U/mL RNasin (Promega), and 100 µM of each amino acid. The final concentration of potassium acetate was adjusted to 100 mM. For standard conditions, the reactions were assembled on ice and then pre-incubated at 25° C for 10 min before adding mRNA. After adding mRNAs, the incubation was continued for an additional 60 min. The 10 min preincubation step was omitted for the experiments in Figures 3A-3C and 5A-5C. Reactions were quenched with four volumes of 1.25x Passive Lysis Buffer (Promega). Pp and Rr luciferase activity was detected in a Monolight 2010 Luminometer (Analytical Luminescence Laboratory) using the Dual-Luciferase Reporter Assay System (Promega).

RNA stability

Reactions with ^{32}P-radiolabeled mRNA were quenched by the addition of 40 volumes of 2x PK buffer (200 mM Tris-HCl, pH 7.5, 25 mM EDTA, 300 mM NaCl, 2% w/v sodium dodecyl sulfate). Proteinase K (E.M. Merck; dissolved in water) was added to a final concentration of 465 µg/ml. The reactions were then incubated for 15 min at 65° C, extracted with phenol/chloroform/isoamyl alcohol (25:24:1), and precipitated with an equal volume of isopropanol. Reactions were analyzed by electrophoresis in a formaldehyde/agarose (0.8% w/v) gel (Sambrook et al., Molecular Cloning. Cold Spring Harbor Laboratory Press, Plainview, NY. (1989)). Radioactivity was detected by exposing the agarose gel [dried under vacuum onto Nytran Plus membrane (Amersham)] to an image plate (Fujix) and quantified using a Fujix Bas 2000 and Image Gauge 3.0 (Fujix) software.

Commercial lysates

Untreated rabbit reticulocyte lysate (Ambion) and wheat germ extract (Ambion) reactions were assembled according to the manufacturer's directions. dsRNA was incubated in the lysate at 27°C (wheat germ) or 30°C (reticulocyte lysate) for 10 min prior to the addition of mRNAs.

Results and Discussion

To evaluate if dsRNA could specifically block gene expression in vitro, reporter mRNAs derived from two different luciferase genes that are unrelated both in sequence and in luciferin substrate specificity were used: Renilla reniformis (sea pansy) luciferase (Rr-Luc) and Photuris pennsylvanica (firefly) luciferase (Pp-Luc). dsRNA generated from one gene was used to target that luciferase mRNA whereas the other luciferase mRNA was an internal control co-translated in the same reaction. dsRNAs of approximately 500 bp were prepared by transcription of polymerase-chain reaction products from the Rr-Luc and Pp-Luc genes. Each dsRNA began ~100 bp downstream of the start of translation (Figure 1). Sense (ss) and anti-sense (as) RNA were transcribed in vitro and annealed to each other to produce the dsRNA. Native gel electrophoresis of the individual Rr 501 and Pp 505 nt as RNA and ssRNA used to form the Rr and Pp dsRNAs was preformed. The ssRNA, asRNA, and dsRNAs were each tested for their ability to block specifically expression of their cognate mRNA but not the expression of the unrelated internal control mRNA.

The ssRNA, asRNA, or dsRNA was incubated for 10 min in a reaction containing Drosophila embryo lysate, then both Pp-Luc and Rr-Luc mRNAs were added and the incubation continued for an additional 60 min. The Drosophila embryo lysate efficiently translates exogenously transcribed mRNA under the conditions used. The amounts of Pp-Luc and Rr-Luc enzyme activities were measured and were used to calculate ratios of either Pp-Luc/Rr-Luc (Figure 2A) or Rr-Luc/Pp-Luc (Figure 2B). To facilitate comparison of different experiments, the ratios from each experiment were normalized to the ratio observed for a control in which buffer was added to the reaction in place of ssRNA, asRNA, or dsRNA.

Figure 2A shows that a 10 nM concentration of the 505 bp dsRNA identical to a portion of the sequence of the Pp-Luc gene specifically inhibited expression of the Pp- Luc mRNA but did not affect expression of the Rr-Luc internal control. Neither ssRNA nor asRNA affected expression of Pp-Luc or the Rr-Luc internal control. Thus, Pp-Luc expression was specifically inhibited by its cognate dsRNA. Conversely, a 10 nM concentration of the 501 bp dsRNA directed against the Rr-Luc mRNA specifically inhibited Rr-Luc expression but not that of the Pp-Luc internal control (Figure 2B). Again, comparable levels of ssRNA or asRNA had little or no effect on expression of either reporter mRNA. On average, dsRNA reduced specific luciferase expression by 70% in these experiments, in which luciferase activity was measured after 1 h incubation. In other experiments in which the translational capacity of the reaction was replenished by the addition of fresh lysate and reaction components, a further reduction in targeted luciferase activity relative to the internal control was observed.

The ability of dsRNA but not asRNA to inhibit gene expression in these lysates is not merely a consequence of the greater stability of the dsRNA (half-life about 2 h) relative to the single-stranded RNAs (half-life ~ 10 min). ssRNA and asRNA transcribed with a 7-methyl guanosine cap were as stable in the lysate as uncapped dsRNA, but do not inhibit gene expression. In contrast, dsRNA formed from the capped ssRNA and asRNA specifically blocks expression of the targeted mRNA.

Effective RNAi in Drosophila requires the injection of about 0.2 fmol of dsRNA into a syncytial blastoderm embryo (Kennerdell and Carthew, Cell 95:1017-1026 (1998); Carthew, www1.pitt.edu/~carthew/manual/RNAi_Protocol.html (1999)). Since the average volume of a Drosophila embryo is approximately 7.3 nl, this corresponds to an intracellular concentration of about 25 nM (Mazur et al., Cryobiology 25:543-544 (1988)). Gene expression in the Drosophila lysate was inhibited by a comparable concentration of dsRNA (10 nM), but lowering the dsRNA concentration ten-fold decreased the amount of specific interference. Ten nanomolar dsRNA corresponds to a 200-fold excess of dsRNA over target mRNA added to the lysate. To test if this excess of dsRNA might reflect a time- and/or concentration-dependent step in which the input dsRNA was converted to a form active for gene-specific interference, the effect of preincubation of the dsRNA on its ability to inhibit expression of its cognate mRNA was examined. Because the translational capacity of the lysates is significantly reduced after 30 min of incubation at 25°C (unpublished observations), it was desired to ensure that all factors necessary for RNAi remained active throughout the pre-incubation period. Therefore, every 30 min, a reaction containing dsRNA and lysate was mixed with a fresh reaction containing unincubated lysate (Figure 3A). After six successive serial transfers spanning 3 hours of preincubation, the dsRNA, now diluted 64-fold relative to its original concentration, was incubated with lysate and 50 pM of target mRNA for 60 min. Finally, the Pp-Luc and Rr-Luc enzyme levels were measured. For comparison, the input amount of dsRNA (10 nM) was diluted 32-fold in buffer, and its capacity to generate gene-specific dsRNA interference in the absence of any preincubation step was assessed.

The preincubation of the dsRNA in lysate significantly potentiated its capacity to inhibit specific gene expression. Whereas the dsRNA diluted 32-fold showed no effect, the preincubated dsRNA was, within experimental error, as potent as undiluted dsRNA, despite having undergone a 64-fold dilution. Potentiation of the dsRNA by preincubation was observed for dsRNAs targeting both the Pp-Luc mRNA (Figure 3B) and the Rr-Luc mRNA (Figure 3C). Taking into account the 64-fold dilution, the activation conferred by preincubation allowed a 156 pM concentration of dsRNA to inhibit 50 pM target mRNA. Further, dilution of the "activated" dsRNA may be effective but has not been tested. We note that although both dsRNAs tested were activated by the preincubation procedure, each fully retained its specificity to interfere with expression only of the mRNA to which it is homologous. Further study of the reactions may provide a route to identifying the mechanism of dsRNA potentiation.

One possible explanation for the observation that preincubation of the dsRNA enhances its capacity to inhibit gene expression in these lysates is that specific factors either modify and/or associate with the dsRNA. Accordingly, the addition of increasing amounts of dsRNA to the reaction might titrate such factors and decrease the amount of gene-specific interference caused by a second dsRNA of unrelated sequence. For both Pp-Luc mRNA and Rr-Luc mRNA, addition of increasing concentrations of the unrelated Drosophila nanos dsRNA to the reaction decreased the amount of gene- specific interference caused by dsRNA targeting the reporter mRNA (Figure 4). None of the tested concentrations of nanos dsRNA affected the levels of translation of the untargeted mRNA, demonstrating that the nanos dsRNA specifically titrated factors involved in gene-specific interference and not components of the translational machinery. The limiting factor(s) was titrated by addition of approximately 1000 nM dsRNA, a 200-fold excess over the 5 nM of dsRNA used to produce specific interference.

Interference in vitro might reflect either a specific inhibition of mRNA translation or the targeted destruction of the specific mRNA. To distinguish these two possibilities, the fates of the Pp-Luc and Rr-Luc mRNAs were examined directly using ^{32}P-radiolabeled substrates. Stability of 10 nM Pp-Luc mRNA or Rr-Luc mRNA incubated in lysate with either buffer or 505 bp Pp-dsRNA (10 nM). Samples were deproteinized after the indicated times and the ^{32}P-radiolabeled mRNAs were then resolved by denaturing gel electrophoresis. In the absence of dsRNA, both the Pp-Luc and Rr-Luc mRNAs were stable in the lysates, with ~ 75%

of the input mRNA remaining after 3 h of incubation. (About 25% of the input mRNA is rapidly degraded in the reaction and likely represents uncapped mRNA generated by the in vitro transcription process.) In the presence of dsRNA (10 nM, 505 bp) targeting the Pp-Luc mRNA, less than 15% of the Pp-Luc mRNA remained after 3 h (Figure 5A). As expected, the Rr-Luc mRNA remained stable in the presence of the dsRNA targeting Pp-Luc mRNA. Conversely, dsRNA (10 nM, 501 bp) targeting the Rr-Luc mRNA caused the destruction of the Rr-Luc mRNA but had no effect on the stability of Pp-Luc mRNA (Figure 5B). Thus, the dsRNA specifically caused accelerated decay of the mRNA to which it is homologous with no effect on the stability of the unrelated control mRNA. This finding indicates that in vivo, at least in Drosophila, the effect of dsRNA is to directly destabilize the target mRNA, not to change the subcellular localization of the mRNA, for example, by causing it to be specifically retained in the nucleus, resulting in non-specific degradation.

These results are consistent with the observation that RNAi leads to reduced cytoplasmic mRNA levels in vivo, as measured by in situ hybridization (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)) and Northern blotting (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998)). Northern blot analyses in trypanosomes and hydra suggest that dsRNA typically decreases mRNA levels by less than 90% (Ngo et al., Proc. Natl. Acad. Sci. USA 95:14687-14692 (1998); Lohmann et al., Dev. Biol. 214:211-214 (1999)). The data presented here show that in vitro mRNA levels are reduced 65 to 85% after three hours incubation, an effect comparable with observations in vivo. They also agree with the finding that RNAi in C. elegans is post- transcriptional (Montgomery et al., Proc. Natl. Acad. Sci. USA 95:15502-15507 (1998)). The simplest explanation for the specific effects on protein synthesis is that it reflects the accelerated rate of RNA decay. However, the results do not exclude independent but specific effects on translation as well as stability.

In vivo, RNAi appears to require a minimum length of dsRNA (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998)). The ability of RNA duplexes of lengths 49 bp, 149 bp, 505 bp, and 997 bp (diagrammed in Figure 1) to target the degradation of the Pp-Luc mRNA in vitro was assessed. In good agreement with in vivo observations, the 49 bp dsRNA was ineffective in vitro, while the 149 bp dsRNA enhanced mRNA decay only slightly, and both the 505 and 997 bp dsRNAs caused robust mRNA degradation (Figure 5C). 50bp dsRNA targeting other portions of the mRNA cause detectable mRNA degradation, though not as robust as that seen for 500bp dsRNA. Thus, although some short dsRNA do not mediate RNAi, others of approximately the same length, but different composition, will be able to do so.

Whether the gene-specific interference observed in Drosophila lysates was a general property of cell-free translation systems was examined. The effects of dsRNAs on expression of Pp-Luc and Rr-Luc mRNA were examined in commercially available wheat germ extracts and rabbit reticulocyte lysates. There was no effect of addition of 10 nM of either ssRNA, asRNA, or dsRNA on the expression of either mRNA reporter in wheat germ extracts. In contrast, the addition of 10 nM of dsRNA to the rabbit reticulocyte lysate caused a profound and rapid, non-specific decrease in mRNA stability. For example, addition of Rr-Luc dsRNA caused degradation of both Rr-Luc and Pp-Luc mRNAs within 15 min. The same non-specific effect was observed upon addition of Pp-Luc dsRNA. The non-specific destruction of mRNA induced by the addition of dsRNA to the rabbit reticulocyte lysate presumably reflects the previously observed activation of RNase L by dsRNA (Clemens and Williams, Cell 13:565-572 (1978); Williams et al., Nucleic Acids Res. 6:1335-1350 (1979); Zhou et al., Cell 72:753-765 (1993); Matthews, Interactions between Viruses and the Cellular Machinery for Protein Synthesis. In Translational Control (eds. J. Hershey, M. Mathews and N. Sonenberg), pp. 505-548. Cold Spring Harbor Laboratory Press, Plainview, NY. (1996)). Mouse cell lines lacking dsRNA-induced anti-viral pathways have recently been described (Zhou et al., Virology 258:435-440 (1999)) and may be useful in the search for mammalian RNAi. Although RNAi is known to exist in some mammalian cells (Wianny and Zernicka-Goetz Nat. Cell Biol. 2: 70-75 (2000)), in many mammalian cell types its presence is likely obscured by the rapid induction by dsRNA of non-specific anti-viral responses.

dsRNA-targeted destruction of specific mRNA is characteristic of RNAi, which has been observed in vivo in many organisms, including Drosophila. The system described above recapitulates in a reaction in vitro many aspects of RNAi. The targeted mRNA is specifically degraded whereas unrelated control mRNAs present in the same solution are not affected. The process is most efficient with dsRNAs greater than 150 bp in length. The dsRNA-specific degradation reaction in vitro is probably general to many, if not all, mRNAs since it was observed using two unrelated genes.

The magnitude of the effects on mRNA stability in vitro described herein are comparable with those reported in vivo (Ngo et al., Proc. Natl. Acad. Sci., USA, 95:14687-14692 (1998); Lohmann et al., Dev. Biol., 214:211-214 (1999). However, the reaction in vitro requires an excess of dsRNA relative to mRNA. In contrast, a few molecules of dsRNA per cell can inhibit gene expression in vivo (Fire et al., Nature, 391: 806-811 (1998); Kennerdell and Carthew, Cell, 95:1017-1026 (1998)). The difference between the stoichiometry of dsRNA to target mRNA in vivo and in vitro should not be surprising in that most in vitro reactions are less efficient than their corresponding in vivo processes. Interestringly, incubation of the dsRNA in the lysate greatly potentiated its activity for RNAi, indicating that it is either modified or becomes associated with other factors or both. Perhaps a small number of molecules is effective in inhibiting the targeted mRNA in vivo because the injected dsRNA has been activated by a process similar to that reported here for RNAi in Drosophila lysates.

Example 2 Double-Stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals

Methods and Material

In vitro RNAi

In vitro RNAi reactions and lysate preparation were as described in Example 1 (Tuschl et al., Genes Dev., 13:3191-7 (1999)) except that the reaction contained 0.03 g/ml creatine kinase, 25 μM creatine phosphate (Fluka), and 1 mM ATP.

Creatine phosphate was freshly dissolved at 500 mM in water for each experiment. GTP was omitted from the reactions, except in Figures 2 and 3.

RNA Synthesis.

Pp-luc and Rr-luc mRNAs and Pp- and Rr-dsRNAs (including dsRNA 'B' in Figure 6) were synthesized by in vitro transcription as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)). To generate transcription templates for dsRNA 'C,' the 5′ sense RNA primer was gcgtaatacgactcactataGAACAAAGGAAACGGATGAT (SEQ ID NO: 2) and the 3′ sense RNA primer was GAAGAAGTTATTCTCCAAAA (SEQ ID NO: 3); the 5′ asRNA primer was gcgtaatacgactcactataGAAGAAGTTATTCTCCAAAA (SEQ ID NO: 4)and the 3′ asRNA primer was GAACAAAGGAAACGGATGAT (SEQ ID NO: 5). For dsRNA 'A' the 5′ sense RNA primer was gcgtaatacgactcactataGTAGCGCGGTGTATTATACC (SEQ ID NO: 6)and the 3′ sense RNA primer was GTACAACGTCAGGTTTACCA (SEQ ID NO: 7); the 5′ asRNA primer was gcgtaatacgactcactataGTACAACGTCAGGTTTACCA (SEQ ID NO: 8)and the 3′ asRNA primer was GTAGCGCGGTGTATTATACC (SEQ ID NO: 9) (lowercase, T7 promoter sequence).

mRNAs were 5′-end-labeled using guanylyl transferase (Gibco/BRL), S-adenosyl methionine (Sigma), and α-^{32}P-GTP (3000 Ci/mmol; New England Nuclear) according to the manufacturer's directions. Radiolabeled RNAs were purified by poly(A) selection using the Poly(A) Tract III kit (Promega). Nonradioactive 7-methyl- guanosine- and adenosine-capped RNAs were synthesized in in vitro transcription reactions with a 5-fold excess of 7-methyl-G(5′)ppp(5′)G or A(5′)ppp(5′)G relative to GTP. Cap analogs were purchased from New England Biolabs.

ATP depletion and Protein Synthesis Inhibition

ATP was depleted by incubating the lysate for 10 minutes at 25°C with 2 mM glucose and 0.1 U/ml hexokinase (Sigma). Protein synthesis inhibitors were purchased from Sigma and dissolved in absolute ethanol as 250-fold concentrated stocks. The final concentrations of inhibitors in the reaction were: anisomycin, 53 mg/ml; cycloheximide, 100 mg/ml; chloramphenicol, 100 mg/ml. Relative protein synthesis was determined by measuring the activity of Rr luciferase protein produced by translation of the Rr-luc mRNA in the RNAi reaction after 1 hour as described previously (Tuschl et al., Genes Dev., 13:3191-7 (1999)).

Analysis of dsRNA Processing

Internally α-^{32}P-ATP-labeled dsRNAs (505 bp Pp-luc or 501 Rr-luc) or 7-methyl-guanosine-capped Rr-luc antisense RNA (501 nt) were incubated at 5 nM final concentration in the presence or absence of unlabeled mRNAs in Drosophila lysate for 2 hours in standard conditions. Reactions were stopped by the addition of 2x proteinase K buffer and deproteinized as described previously (Tuschl et al., Genes Dev., 13:3191- 3197 (1999)). Products were analyzed by electrophoresis in 15% or 18% polyacrylamide sequencing gels. Length standards were generated by complete RNase T1 digestion of α-^{32}P-ATP-labeled 501 nt Rr-luc sense RNA and asRNA.

For analysis of mRNA cleavage, 5′-^{32}P-radiolabeled mRNA (described above) was incubated with dsRNA as described previously (Tuschl et al., Genes Dev., 13:3191- 3197 (1999)) and analyzed by electrophoresis in 5% (Figure 5B) and 6% (Figure 6C) polyacrylamide sequencing gels. Length standards included commercially available RNA size standards (FMC Bioproducts) radiolabeled with guanylyl transferase as described above and partial base hydrolysis and RNase T1 ladders generated from the 5′-radiolabeled mRNA.

Deamination Assay

Internally α-^{32}P-ATP-labeled dsRNAs (5 nM) were incubated in Drosophila lysate for 2 hours at standard conditions. After deproteinization, samples were run on 12% sequencing gels to separate full-length dsRNAs from the 21-23 nt products. RNAs were eluted from the gel slices in 0.3 M NaCl overnight, ethanol-precipitated, collected by centrifugation, and redissolved in 20 µl water. The RNA was hydrolyzed into nucleoside 5 -phosphates with nuclease P1 (10 µl reaction containing 8 µl RNA in water, 30 mM KOAc pH 5.3, 10 mM $ZnSO_4$, 10 µg or 3 units nuclease P1, 3 hours, 50° C). Samples (1 ml) were co-spotted with non-radioactive 5 -mononucleotides [0.05 O.D. units (A_{260}) of pA, pC, pG, pI, and pU] on cellulose HPTLC plates (EM Merck) and separated in the first dimension in isobutyric acid/25% ammonia/water (66/1/33, v/v/v) and in the second dimension in 0.1M sodium phosphate, pH 6.8/ammonium sulfate/1-propanol (100/60/2, v/w/v; Silberklang et al., 1979). Migration of the non- radioactive internal standards was determined by UV-shadowing.

Results and Discussion

RNAi Requires ATP

As described in Example 1, Drosophila embryo lysates faithfully recapitulate RNAi (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Previously, dsRNA-mediated gene silencing was monitored by measuring the synthesis of luciferase protein from the targeted mRNA. Thus, these RNAi reactions contained an ATP-regenerating system, needed for the efficient translation of the mRNA. To test if ATP was, in fact, required for RNAi, the lysates were depleted for ATP by treatment with hexokinase and glucose, which converts ATP to ADP, and RNAi was monitored directly by following the fate of ^{32}P-radiolabeled Renilla reniformis luciferase (Rr-luc) mRNA (Figure 6). Treatment with hexokinase and glucose reduced the endogenous ATP level in the lysate from 250 μM to below 10 μM. ATP regeneration required both exogenous creatine phosphate and creatine kinase, which acts to transfer a high-energy phosphate from creatine phosphate to ADP. When ATP-depleted extracts were supplemented with either creatine phosphate or creatine kinase separately, no RNAi was observed. Therefore, RNAi requires ATP in vitro. When ATP, creatine phosphate, and creatine kinase were all added together to reactions containing the ATP-depleted lysate, dsRNA-dependent degradation of the Rr-luc mRNA was restored (Figure 6). The addition of exogenous ATP was not required for efficient RNAi in the depleted lysate, provided that both creatine phosphate and creatine kinase were present, demonstrating that the endogenous concentration (250 mM) of adenosine nucleotide is sufficient to support RNAi. RNAi with a Photinus pyralis luciferase (Pp-luc) mRNA was also ATP-dependent.

The stability of the Rr-luc mRNA in the absence of Rr-dsRNA was reduced in ATP-depleted lysates relative to that observed when the energy regenerating system was included, but decay of the mRNA under these conditions did not display the rapid decay kinetics characteristic of RNAi in vitro, nor did it generate the stable mRNA cleavage products characteristic of dsRNA-directed RNAi. These experiments do not establish if the ATP requirement for RNAi is direct, implicating ATP in one or more steps in the RNAi mechanism, or indirect, reflecting a role for ATP in maintaining high concentrations of another nucleoside triphosphate in the lysate.

Translation Is Not Required for RNAi In Vitro

The requirement for ATP suggested that RNAi might be coupled to mRNA translation, a highly energy-dependent process. To test this possibility, various inhibitors of protein synthesis were added to the reaction by preparing a denaturing agarose-gel analysis of 5′-32P-radiolabeled Pp-luc mRNA after incubation for indicated times in a standard RNAi reaction with and without protein synthesis inhibitors. The eukaryotic translation inhibitors anisomycin, an inhibitor of initial peptide bond formation, cycloheximide, an inhibitor of peptide chain elongation, and puromycin, a tRNA mimic which causes premature termination of translation (Cundliffe, Antibiotic Inhibitors of Ribosome Function. In The Molecular Basis of Antibiotic Action, E. Gale, E. Cundliffe, P. Reynolds, M. Richmond and M. Warning, eds. (New York: Wiley), pp. 402-547. (1981)) were tested. Each of these inhibitors reduced protein synthesis in the Drosophila lysate by more than 1,900-fold (Figure 7A). In contrast, chloramphenicol, an inhibitor of Drosophila mitochondrial protein synthesis (Page and Orr-Weaver, Dev. Biol., 183:195-207 (1997)), had no effect on translation in the lysates (Figure 7A). Despite the presence of anisomycin, cycloheximide, or chloramphenicol, RNAi proceeded at normal efficiency. Puromycin also did not perturb efficient RNAi. Thus, protein synthesis is not required for RNAi in vitro.

Translational initiation is an ATP-dependent process that involves recognition of the 7-methyl guanosine cap of the mRNA (Kozak, Gene, 234:187-208

(1999); Merrick and Hershey, The Pathway and Mechanism of Eukaryotic Protein Synthesis. In Translational Control, J. Hershey, M. Mathews and N. Sonenberg, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 31-69 (1996)). The Drosophila lysate used to support RNAi in vitro also recapitulates the cap-dependence of translation; Pp-luc mRNA with a 7-methyl-guanosine cap was translated greater than ten-fold more efficiently than was the same mRNA with an A(5′)ppp(5′)G cap (Figure 7B). Both RNAs were equally stable in the Drosophila lysate, showing that this difference in efficiency cannot be merely explained by more rapid decay of the mRNA with an adenosine cap (see also Gebauer et al., EMBO J., 18:6146-54 (1999)). Although the translational machinery can discriminate between Pp-luc mRNAs with 7- methyl-guanosine and adenosine caps, the two mRNAs were equally susceptible to RNAi in the presence of Pp-dsRNA (Figure 7C). These results suggest that steps in cap recognition are not involved in RNAi.

dsRNA Is Processed to 21-23 nt Species

RNAs 25 nt in length are generated from both the sense and anti-sense strands of genes undergoing post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Denaturing acrylamide-gel analysis of the products formed in a two-hour incubation of uniformly ^{32}P-radiolabeled dsRNAs and capped asRNA in lysate under standard RNAi conditions, in the presence or absence of target mRNAs. It was found that dsRNA is also processed to small RNA fragments. When incubated in lysate, approximately 15% of the input radioactivity of both the 501 bp Rr-dsRNA and the 505 bp Pp-dsRNA appeared in 21 to 23 nt RNA fragments. Because the dsRNAs are more than 500 bp in length, the 15% yield of fragments implies that multiple 21-23 nt RNAs are produced from each full-length dsRNA molecule. No other stable products were detected. The small RNA species were produced from dsRNAs in which both strands were uniformly ^{32}P-radiolabeled. Formation of the 21-23 nt RNAs from the dsRNA did not require the presence of the corresponding mRNA, demonstrating that the small RNA species is generated by processing of the dsRNA, rather than as a product of dsRNA-targeted mRNA degradation. It was noted that 22 nucleotides corresponds to two turns of an A-form RNA-RNA helix.

When dsRNAs radiolabeled within either the sense or the anti-sense strand were incubated with lysate in a standard RNAi reaction, 21-23 nt RNAs were generated with comparable efficiency. These data support the idea that the 21-23 nt RNAs are generated by symmetric processing of the dsRNA. A variety of data support the idea that the 21-23 nt RNA is efficiently generated only from dsRNA and is not the consequence of an interaction between single-stranded RNA and the dsRNA. First, a ^{32}P-radiolabeled 505 nt Pp-luc sense RNA or asRNA was not efficiently converted to the 21-23 nt product when it was incubated with 5 nM nonradioactive 505 bp Pp- dsRNA. Second, in the absence of mRNA, a 501 nt 7-methyl-guanosine-capped Rr- asRNA produced only a barely detectable amount of 21-23 nt RNA (capped single- stranded RNAs are as stable in the lysate as dsRNA, Tuschl et al., Genes Dev., 13:3191- 7 (1999)), probably due to a small amount of dsRNA contaminating the anti-sense preparation. However, when Rr-luc mRNA was included in the reaction with the ^{32}P- radiolabeled, capped Rr-asRNA, a small amount of 21-23 nt product was generated, corresponding to 4% of the amount of 21-23 nt RNA produced from an equimolar amount of Rr-dsRNA. This result is unlikely to reflect the presence of contaminating dsRNA in the Rr-asRNA preparation, since significantly more product was generated from the asRNA in the presence of the Rr-luc mRNA than in the absence. Instead, the data suggest that asRNA can interact with the complementary mRNA sequences to form dsRNA in the reaction and that the resulting dsRNA is subsequently processed to the small RNA species. Rr-asRNA can support a low level of bona fide RNAi in vitro (see below), consistent with this explanation.

It was next asked if production of the 21-23 nt RNAs from dsRNA required ATP. When the 505 bp Pp-dsRNA was incubated in a lysate depleted for ATP by treatment with hexokinase and glucose, 21-23 nt RNA was produced, albeit 6 times slower than when ATP was regenerated in the depleted lysate by the inclusion of creatine kinase and creatine phosphate. Therefore, ATP may not be required for production of the 21-23 nt RNA species, but may instead simply enhance its formation. Alternatively, ATP may be required for processing of the dsRNA, but at a concentration less than that remaining after hexokinase treatment. The molecular basis for the slower mobility of the small RNA fragments generated in the ATP-depleted lysate is not understood.

Wagner and Sun (Wagner and Sun, Nature, 391:744-745 (1998)) and Sharp (Sharp, Genes Dev., 13:139-41 (1999)) have speculated that the requirement for dsRNA in gene silencing by RNAi reflects the involvement of a dsRNA-specific adenosine deaminase in the process. dsRNA adenosine deaminases unwind dsRNA by converting adenosine to inosine, which does not base-pair with uracil. dsRNA adenosine deaminases function in the post-transcriptional editing of mRNA (for review see Bass, Trends Biochem. Sci., 22:157-62 (1997)). To test for the involvement of dsRNA adenosine deaminase in RNAi, the degree of conversion of adenosine to inosine in the 501 bp Rr-luc and 505 bp Pp-luc dsRNAs after incubation with Drosophila embryo lysate in a standard in vitro RNAi reaction was examined. Adenosine deamination in full-length dsRNA and the 21-23 nt RNA species was assessed by two-dimensional thin-layer chromatography. Inorganic phosphate ($P_{i,}$) was produced by the degradation of mononucleotides by phosphatases that contaminate commercially available nuclease P1 (Auxilien et al., J. Mol. Biol., 262:437-458 (1996)). The degree of adenosine deamination in the 21-23 nt species was also determined. The full-length dsRNA radiolabeled with [^{32}P]-adenosine was incubated in the lysate, and both the full-length dsRNA and the 21-23 nt RNA products were purified from a denaturing acrylamide gel, cleaved to mononucleotides with nuclease P1, and analyzed by two-dimensional thin- layer chromatography.

A significant fraction of the adenosines in the full-length dsRNA were converted to inosine after 2 hours (3.1% and 5.6% conversion for Pp-luc and Rr-luc dsRNAs, respectively). In contrast, only 0.4% (Pp-dsRNA) or 0.7% (Rr-dsRNA) of the adenosines in the 21-23 nt species were deaminated. These data imply that fewer than 1 in 27 molecules of the 21-23 nt RNA species contain an inosine. Therefore, it is unlikely that dsRNA-dependent adenosine deamination within the 21-23 nt species is required for its production.

asRNA Generates a Small Amount of RNAi in vitro

When mRNA was ^{32}P-radiolabeled within the 5′-7-methyl-guanosine cap, stable 5′ decay products accumulated during the RNAi reaction. Such stable 5′decay products were observed for both the Pp-luc and Rr-luc mRNAs when they were incubated with their cognate dsRNAs. Previously, it was reported that efficient RNAi does not occur when asRNA is used in place of dsRNA (Tuschl et al., Genes Dev., 13:3191-7 (1999)). Nevertheless, mRNA was measurably less stable when incubated with asRNA than with buffer (Figures 8A and 8B). This was particularly evident for the Rr-luc mRNA: approximately 90% of the RNA remained intact after a 3-hour incubation in lysate, but only 50% when asRNA was added. Less than 5% remained when dsRNA was added. Interestingly, the decrease in mRNA stability caused by asRNA was accompanied by the formation of a small amount of the stable 5′-decay products characteristic of the RNAi reaction with dsRNA. This finding parallels the observation that a small amount of 21- 23 nt product formed from the asRNA when it was incubated with the mRNA (see above) and lends strength to the idea that asRNA can enter the RNAi pathway, albeit inefficiently.

mRNA Cleavage Sites Are Determined by the Sequence of the dsRNA

The sites of mRNA cleavage were examined using three different dsRNAs, 'A,' 'B,' and 'C,' displaced along the Rr-luc sequence by approximately 100 nts. Denaturing acrylamide-gel analysis of the stable, 5′-cleavage products produced after incubation of the Rr-luc mRNA for the indicated times with each of the three dsRNAs, 'A,' 'B,' and 'C,' or with buffer (Ø) was performed. The positions of these relative to the Rr-luc mRNA sequence are shown in Figure 9. Each of the three dsRNAs was incubated in a standard RNAi reaction with Rr-luc mRNA ^{32}P-radiolabeled within the 5′-cap. In the absence of dsRNA, no stable 5′-cleavage products were detected for the mRNA, even after 3 hours of incubation in lysate. In contrast, after a 20-minute incubation, each of the three dsRNAs produced a ladder of bands corresponding to a set of mRNA cleavage products characteristic for that particular dsRNA. For each dsRNA, the stable, 5′ mRNA cleavage products were restricted to the region of the Rr-luc mRNA that corresponded to the dsRNA

(Figures 9 and 10). For dsRNA 'A,' the lengths of the 5′- cleavage products ranged from 236 to just under ~750 nt; dsRNA 'A' spans nucleotides 233 to 729 of the Rr-luc mRNA. Incubation of the mRNA with dsRNA 'B' produced mRNA 5′-cleavage products ranging in length from 150 to ~600 nt; dsRNA 'B' spans nucleotides 143 to 644 of the mRNA. Finally, dsRNA 'C' produced mRNA cleavage products from 66 to ~500 nt in length. This dsRNA spans nucleotides 50 to 569 of the Rr-luc mRNA. Therefore, the dsRNA not only provides specificity for the RNAi reaction, selecting which mRNA from the total cellular mRNA pool will be degraded, but also determines the precise positions of cleavage along the mRNA sequence.

The mRNA Is Cleaved at 21-23 Nucleotide Intervals

To gain further insight into the mechanism of RNAi, the positions of several mRNA cleavage sites for each of the three dsRNAs were mapped (Figure 10). High resolution denaturing acrylamide-gel analysis of a subset of the 5′-cleavage products described above was performed. Remarkably, most of the cleavages occurred at 21-23 nt intervals (Figure 10). This spacing is especially striking in light of our observation that the dsRNA is processed to a 21-23 nt RNA species and the finding of Hamilton and Baulcombe that a 25 nt RNA correlates with post-transcriptional gene silencing in plants (Hamilton and Baulcombe, Science, 286:950-2 (1999)). Of the 16 cleavage sites we mapped (2 for dsRNA 'A,' 5 for dsRNA 'B,' and 9 for dsRNA 'C'), all but two reflect the 21-23 nt interval. One of the two exceptional cleavages was a weak cleavage site produced by dsRNA 'C' (indicated by an open blue circle in Figure 10). This cleavage occurred 32 nt 5′ to the next cleavage site. The other exception is particularly intriguing. After four cleavages spaced 21-23 nt apart, dsRNA 'C' caused cleavage of the mRNA just nine nt 3′ to the previous cleavage site (red arrowhead in Figure 10). This cleavage occurred in a run of seven uracil residues and appears to "reset" the ruler for cleavage; the next cleavage site was 21-23 nt 3′ to the exceptional site. The three subsequent cleavage sites that we mapped were also spaced 21-23 nt apart. Curiously, of the sixteen cleavage sites caused by the three different dsRNAs, fourteen occur at uracil residues. The significance of this finding is not understood, but it suggests that mRNA cleavage is determined by a process which measures 21-23 nt intervals and which has a sequence preference for cleavage at uracil. Results show that the 21-23 nt RNA species produced by incubation of ~500 bp dsRNA in the lysate caused sequence-specific interference in vitro when isolated from an acrylamide gel and added to a new RNAi reaction in place of the full-length dsRNA.

A Model for dsRNA-directed mRNA Cleavage

Without wishing to be bound by theory, the biochemical data described herein, together with recent genetic experiments in C. elegans and Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999); Grishok et al., Science, 287: 2494-7 (2000); Ketting et al., Cell, 99:133-41 (1999); Tabara et al., Cell, 99:123-32 (1999)), suggest a model for how dsRNA targets mRNA for destruction (Figure 11). In this model, the dsRNA is first cleaved to 21-23 nt long fragments in a process likely to involve genes such as the C. elegans loci rde-1 and rde-4. The resulting fragments, probably as short asRNAs bound by RNAi-specific proteins, would then pair with the mRNA and recruit a nuclease that cleaves the mRNA. Alternatively, strand exchange could occur in a protein-RNA complex that transiently holds a 21-23 nt dsRNA fragment close to the mRNA. Separation of the two strands of the dsRNA following fragmentation might be assisted by an ATP-dependent RNA helicase, explaining the observed ATP enhancement of 21-23 nt RNA production.

It is likely that each small RNA fragment produces one, or at most two, cleavages in the mRNA, perhaps at the 5′ or 3′ ends of the 21-23 nt fragment. The small RNAs may be amplified by an RNA-directed RNA polymerase such as that encoded by the ego-1 gene in C. elegans (Smardon et al., Current Biology, 10:169-178 (2000)) or the qde-1 gene in Neurospora (Cogoni and Macino, Nature, 399:166-9 (1999)), producing long-lasting post-transcriptional gene silencing in the absence of the dsRNA that initiated the RNAi effect. Heritable RNAi in C. elegans requires the rde-1 and rde-4 genes to initiate, but not to persist in subsequent generations. The rde-2, rde- 3, and mut-7 genes in C. elegans are required in the tissue where RNAi occurs, but are not required for initiation of heritable RNAi

(Grishok et al., Science, in press 2000). These 'effector' genes (Grishok et al., Science, in press 2000) are likely to encode proteins functioning in the actual selection of mRNA targets and in their subsequent cleavage. ATP may be required at any of a number of steps during RNAi, including complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21-23 nt RNAs with the target mRNA, mRNA cleavage, and recycling of the targeting complex. Testing these ideas with the in vitro RNAi system will be an important challenge for the future. Some genes involved in RNAi are also important for transposon silencing and co-suppresion. Co-suppression is a broad biological phenomenon spanning plants, insects and perhaps humans. The most likely mechanism in Drosophila melanogaster is transcriptional silencing (Pal-Bhanra et al, Cell 99: 35-36. Thus, 21-23 nt fragments are likely to be involved in transcriptional control, as well as in post-transcriptional cotrol.

Example 3 Isolated 21-23 mers caused sequence-specific interference when added to a new RNAi reaction

Isolation of 21-23 nt fragments from incubation reaction of 500 bp dsRNA in lysate.

Double-stranded RNA (500 bp from) was incubated at 10 nM concentration in Drosophila embryo lysate for 3 h at 25° C under standard conditions as described herein. After deproteinization of the sample, the 21-23 nt reaction products were separated from unprocessed dsRNA by denaturing polyacrylamide (15%) gel electrophoresis. For detection of the non-radiolabeled 21-23 nt fragments, an incubation reaction with radiolabeled dsRNA was loaded in a separate lane of the same gel. Gel slices containing the non-radioactive 21-23 nt fragments were cut out and the 21-23 nt fragments were eluted from the gel slices at 4° C overnight in 0.4 ml 0.3 M NaCl. The RNA was recovered from the supernatant by ethanol precipitation and centrifugation. The RNA pellet was dissolved in 10 µl of lysis buffer. As control, gel slices slightly above and below the 21-23 nt band were also cut out and subjected to the same elution and precipitation procedures. Also, a non-incubated dsRNA loaded on the 15% gel and a gel slice corresponding to 21-23 nt fragments was cut out and eluted. All pellets from the control experiments were dissolved in 10 µl lysis buffer. The losses of RNA during recovery from gel slices by elution are approx. 50%.

Incubation of purified 21-23 nt fragments in a translation-based RNAi assay

1 µl of the eluted 21-23 mer or control RNA solution was used for a standard 10 µl RNAi incubation reaction (see above). The 21-23 mers were preincubated in the lysate containing reaction mixture for 10 or 30 min before the addition of the target and control mRNA. During pre-incubation, proteins involved in RNA interference may re- associate with the 21-23 mers due to a specific signal present on these RNAs. The incubation was continued for another hour to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The RNA interference is the expressed as the ratio of target to control luciferase activity normalized by an RNA-free buffer control. Specific suppression of the target gene was observed with either 10 or 30 minutes pre- incubation. The suppression was reproducible and reduced the relative ratio of target to control by 2-3 fold. None of the RNA fragments isolated as controls showed specific interference. For comparison, incubation of 5 nM 500 bp dsRNA (10 min pre- incubation) affects the relative ratio of control to target gene approx. 30-fold.

Stability of isolated 21-23 nt fragments in a new lysate incubation reaction.

Consistent with the observation of RNAi mediated by purified 21-23 nt RNA fragment, it was found that 35% of the input 21-23 nt RNA persists for more than 3 h in such an incubation reaction. This suggests that cellular factors associate with the deproteinized 21-23 nt fragments and reconstitute a functional mRNA-degrading particle. Signals connected with these 21-23 nt fragments, or their possible double stranded nature or specific lengths are likely responsible for this observation. The 21-23 nt fragments have a terminal 3' hydroxyl group, as evidenced by altered mobility on a sequencing gel following periodate treatment and beta-elimination.

Example 4 21-23-mers purified by non-denaturing methods caused sequence-specific interference when added to a new RNAi reaction.

Fifty nanomolar double-stranded RNA (501 bp Rr-luc dsRNA, as described in example 1) was incubated in a 1 ml in vitro reaction with lysate at 25°C (see example 1). The reaction was then stopped by the addition of an equal volume of 2x PK buffer (see example 1) and proteinase K was added to a final concentration of 1.8 µg/µl. The reaction was incubated for an additional 1 h at 25°C, phenol extracted, and then the RNAs were precipitated with 3 volumes of ethanol. The ethanol precipitate was collected by centrifugation, and the pellet was resuspended in 100 µl of lysis buffer and applied to a Superdex HR 200 10/30 gel filtration column (Pharmacia) run in lysis buffer at 0.75 ml/min. 200 µl fractions were collected from the column. Twenty µl of 3 M sodium acetate and 20 µg glycogen was added to each fraction, and the RNA was recovered by precipitation with 3 volumes of ethanol. The precipitates were resuspended in 30 µl of lysis buffer. Column profiles following the fractionation of 32P-labeled input RNA are shown in Figure 13A.

One microliter of each resuspended fraction was tested in a 10 µl standard in vitro RNAi reaction (see example 1). This procedure yields a concentration of RNA in the in vitro RNAi reaction that is approximately equal to the concentration of that RNA species in the original reaction prior to loading on the column. The fractions were preincubated in the lysate containing reaction mixture for 30 min before the addition of 10 nM Rr-luc mRNA target and 10 nM Pp-luc control mRNA. During pre-incubation, proteins involved in RNA interference may re-associate with the 21-23-mers due to a specific signal present on these RNAs. The incubation was continued for another three hours to allow translation of the target and control mRNAs. The reaction was quenched by the addition of passive lysis buffer (Promega), and luciferase activity was measured. The suppression of Rr-luc mRNA target expression by the purified 21-23 nt fragments was reproducible and reduced the relative ratio of target to control by >30-fold, an amount comparable to a 50 nM 500 bp dsRNA control. Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed.

The data show that the both the fractions containing uncleaved dsRNA (fractions 3 - 5) or long, partially cleaved dsRNA (fractions 7 - 13) and the fractions containing the fully processed 21-23 nt siRNAs (fractions 41 - 50) mediate effective RNA interference in vitro (Figure 13B). Suppression of target mRNA expression was specific: little or no effect on the expression of the Pp-luc mRNA control was observed (Figure 13C). These data, together with those in the earlier examples, demonstrate that the 21-23 nt siRNAs are (1) true intermediates in the RNAi pathway and (2) effective mediators of RNA interference in vitro.

Example 5 21-nucleotide siRNA duplexes mediate RNA interference in human tissue cultures

Methods

RNA preparation

21 nt RNAs were chemically synthesized using Expedite RNA phosphoramidites and thymidine phosphoramidite (Proligo, Germany). Synthetic oligonucleotides were deprotected and gel-purified (Elbashir, S.M., Lendeckel, W. & Tuschl, T., *Genes & Dev. 15,* 188-200 (2001)), followed by Sep-Pak C18 cartridge (Waters, Milford, MA, USA) purification (Tuschl, t., *et al., Biochemistry, 32:*11658-11668 (1993)). The siRNA sequences targeting GL2 (Acc. X65324) and GL3 luciferase (Acc. U47296) corresponded to the coding regions 153-173 relative to the first nucleotide of the start codon, siRNAs targeting RL (Acc. AF025846) corresponded to region 119-129 after the start codon. Longer RNAs were transcribed with T7 RNA polymerase from PCR products, followed by gel and Sep-Pak purification. The 49 and 484 bp GL2 or GL3 dsRNAs corresponded to position 113-161 and 113-596, respectively, relative to the start of translation; the 50 and 501 bp RL dsRNAs corresponded to position 118-167 and 118-618, respectively. PCR templates for dsRNA synthesis targeting humanized GFP (hG) were amplified from pAD3 (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)), whereby 50 and 501 bp hG dsRNA corresponded to position 118-167 and 118-618, respectively, to the start codon.

For annealing of siRNAs, 20 µM single strands were incubated in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH at pH 7.4, 2 mM magnesium acetate) for 1 min at 90°C followed by 1 h at 37 °C. The 37 °C incubation step was extended overnight for the 50 and 500 bp dsRNAs, and these annealing reactions were performed at 8.4 µM and 0.84 µM strand concentrations, respectively.

Cell culture

S2 cells were propagated in Schneider's *Drosophila* medium (Life Technologies) supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin at 25 °C. 293, NIH/3T3, HeLa S3, COS-7 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin. Cells were regularly passaged to maintain exponential growth. 24 h before transfection at approx. 80% confluency, mammalian cells were trypsinized and diluted 1:5 with fresh medium without antibiotics (1-3 × 10^5 cells/ml) and transferred to 24-well plates (500 µl/well). S2 cells were not trypsinized before splitting. Transfection was carried out with Lipofectamine 2000 reagent (Life Technologies) as described by the manufacturer for adherent cell lines. Per well, 1.0 µg pGL2-Control (Promega) or pGL3-Control (Promega), 0.1 µg pRL-TK (Promega), and 0.28 µg siRNA duplex or dsRNA, formulated into liposomes, were applied; the final volume was 600 µl per well. Cells were incubated 20 h after transfection and appeared healthy thereafter. Luciferase expression was subsequently monitored with the Dual luciferase assay (Promega). Transfection efficiencies were determined by fluorescence microscopy for mammalian cell lines after co-transfection of 1.1 µg hGFP-encoding pAD3[22] and 0.28 µg invGL2 siRNA, and were 70-90%. Reporter plasmids were amplified in XL-1 Blue (Strategene) and purified using the Qiagen EndoFree Maxi Plasmid Kit.

Results

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing in animals and plants, initiated by double-stranded

RNA (dsRNA) homologous in sequence to the silenced gene (Fire, A., *Trends Genet., 15:*358-363 (1999); Sharp, P.A. & Zamore, P.D., *Science, 287:*2431-2433 (2000); Sijen, T. & Kooter, J.M., *Bioessays, 22:*520-531 (2000); Bass, B.L., *Cell, 101:*235-238 (2000); Hammond, S.M., *et al., Nat. Rev. Genet., 2:*110-119 (2001)). The mediators of sequence-specific mRNA degradation are 21 and 22 nt small interfering RNAs (siRNAs) generated by RNase III cleavage from longer dsRNAs[6-10] (Hamilton, A.J. &Baulcombe, D.C., *Science, 286:*950-952 (1999); Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Bernstein, E., *et al., Naature, 409:*363-366 (2001); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). As shown herein, 21 nt siRNA duplexes are able to specifically suppress reporter gene expression in multiple mammalian tissue cultures, including human embryonic kidney (293) and HeLa cells. In contrast to 50 or 500 bp dsRNAs, siRNAs do not activate the interferon response. These results indicate that siRNA duplexes are a general tool for sequence-specific inactivation of gene function in mammalian cells.

Base-paired 21 and 22 nt siRNAs with overhanging 3' ends mediate efficient sequence-specific mRNA degradation in lysates prepared from *D. melanogaster* embryos (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). To test whether siRNAs are also capable of mediating RNAi in tissue culture, 21 nt siRNA duplexes with symmetric 2 nt 3' overhangs directed against reporter genes coding for sea pansy (*Renilla reniformis*) and two sequence variants of firefly (*Photinus pyralis*, GL2 and GL3) luciferases (Figures 14A, 14B) were constructed. The siRNA duplexes were co-transfected with the reporter plasmid combinations pGL2/pRL or pGL3/pRL, into *D. melanogaster Schneider S2* cells or mammalian cells using cationic liposomes. Luciferase activities were determined 20 h after transfection. In all cell lines tested, specific reduction of the expression of the reporter genes in the presence of cognate siRNA duplexes was observed (Figures 15A-15J). Remarkably, the absolute luciferase expression levels were unaffected by non-cognate siRNAs, indicating the absence of harmful side effects by 21 nt RNA duplexes (e.g. Figures 16A-16D, for HeLa cells). In *D. melanogaster* S2 cells (Figures 15A, 15B), the specific inhibition of luciferases was complete, and similar to results previously obtained for longer dsRNAs (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Caplen, N.J., *et al., Gene, 252:*95-105 (2000); Clemens, M & Williams, B., *Cell, 13:*565-572 (1978); Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). In mammalian cells, where the reporter genes were 50- to 100-fold stronger expressed, the specific suppression was less complete (Figures 15C-15J). GL2 expression was reduced 3- to 12-fold, GL3 expression 9- to 25-fold, and RL expression 1- to 3-fold, in response to the cognate siRNAs. For 293 cells, targeting of RL luciferase by RL siRNAs was ineffective, although GL2 and GL3 targets responded specifically (Figures 15I, 15J). It is likely that the lack of reduction of RL expression in 293 cells is due to its 5- to 20-fold higher expression compared to any other mammalian cell line tested and/or to limited accessibility of the target sequence due to RNA secondary structure or associated proteins. Nevertheless, specific targeting of GL2 and GL3 luciferase by the cognate siRNA duplexes indicated that RNAi is also functioning in 293 cells.

The 2 nt 3' overhang in all siRNA duplexes, except for uGL2, was composed of (2'-deoxy) thymidine. Substitution of uridine by thymidine in the 3' overhang was well tolerated in the *D. melanogaster* in vitro system, and the sequence of the overhang was uncritical for target recognition (Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). The thymidine overhang was chosen, because it is supposed to enhance nuclease resistance of siRNAs in the tissue culture medium and within transfected cells. Indeed, the thymidine-modified GL2 siRNA was slightly more potent than the unmodified uGL2 siRNA in all cell lines tested (Figures 15A, 15C, 15E, 15G, 15I). It is conceivable that further modifications of the 3' overhanging nucleotides will provide additional benefits to the delivery and stability of siRNA duplexes.

In co-transfection experiments, 25 nM siRNA duplexes with respect to the final volume of tissue culture medium were used (Figures 15A-15J, 16A-16F). Increasing the siRNA concentration to 100 nM did not enhance the specific silencing effects, but started to affect transfection efficiencies due to competition for liposome encapsulation between plasmid DNA and siRNA. Decreasing the siRNA concentration to 1.5 nM did not reduce the specific silencing effect, even though the siRNAs were now only 2- to 20-fold more concentrated than the DNA plasmids. This indicates that siRNAs are extraordinarily powerful reagents for mediating gene silencing, and that siRNAs are effective at concentrations that are several orders of magnitude below the concentrations applied in conventional antisense or ribozyme gene targeting experiments.

In order to monitor the effect of longer dsRNAs on mammalian cells, 50 and 500 bp dsRNAs cognate to the reporter genes were prepared. As non-specific control, dsRNAs from humanized GFP (hG) (Kehlenbach, R.H., *et al., J. Cell Biol., 141:*863-874 (1998)) was used. When dsRNAs were co-transfected, in identical amounts (not concentrations) to the siRNA duplexes, the reporter gene expression was strongly and unspecifically reduced. This effect is illustrated for HeLa cells as a representative example (Figures 16A-16D). The absolute luciferase activities were decreased unspecifically 10- to 20-fold by 50 bp dsRNA, and 20- to 200-fold by 500 bp dsRNA co-transfection, respectively. Similar unspecific effects were observed for COS-7 and NIH/3T3 cells. For 293 cells, a 10- to 20-fold unspecific reduction was observed only for 500 bp dsRNAs. Unspecific reduction in reporter gene expression by dsRNA > 30 bp was expected as part of the interferon response (Matthews, M., Interactions between viruses and the cellular machinery for protein synthesis in *Translational Control* (eds., Hershey, J., Matthews,M. & Sonenberg, N.) 505-548 (Cold Spring Harbor Laboratory Press, Plainview, NY; 1996); Kumar, M. & Carmichael, G.G., *Microbiol. Mol. Biol. Rev., 62:*1415-1434 (1998); Stark, G.R., *et al., Annu. Rev. Biochem., 67:*227-264 (1998)). Surprisingly, despite the strong unspecific decrease in reporter gene expression, additional sequence-specific, dsRNA-mediated silencing were reproducibly detected. The specific silencing effects, however, were only apparent when the relative reporter gene activities were normalized to the hG dsRNA controls (Figures 16E, 16F). A 2- to 10-fold specific reduction in response to cognate dsRNA was observed, also in the other three mammalian cell lines tested. Specific silencing effects with dsRNAs (356-1662 bp) were previously reported in CHO-K1 cells, but the amounts of dsRNA required to detect a 2- to 4-fold specific reduction were about 20-fold higher than in our experiments (Ui-Tei, K., *et al., FEBS Letters, 479:*79-82 (2000)). Also, CHO-K1 cells appear to be deficient in the interferon response. In another report, 293, NIH/3T3, and BHK-21 cells were tested for RNAi using luciferase/lacZ reporter combinations and 829 bp specific lacZ or 717 bp unspecific GFP dsRNA (Caplen, N.J., *et al., Gene, 252:*95-105 (2000)). The failure of detecting RNAi in this case is likely due to the less sensitive luciferase/lacZ reporter assay and the length differences of target and control dsRNA. Taken together, the results described herein indicate that RNAi is active in mammalian cells, but that the silencing effect is difficult to detect if the interferon system is activated by dsRNA >30 bp.

The mechanism of the 21 nt siRNA-mediated interference process in mammalian cells remains to be uncovered, and silencing may occur post-transcriptional and/or transcriptional. In *D. melanogaster* lysate, siRNA duplexes mediate post-transcriptional gene silencing by reconstitution of a siRNA-protein complexes (siRNPs), which are guiding mRNA recognition and targeted cleavage (Hammond, S.M., *et al., Nature, 404:*293-296 (2000); Zamore, P.D., *et al., Cell, 101:*25-33 (2000); Elbashir, S.M., *et al., Genes & Dev., 15:*188-200 (2001)). In plants, dsRNA-mediated post-transcriptional silencing has also been linked to RNA-directed DNA methylation, which may also be directed by 21 nt siRNAs (Wassenegger, M., *Plant Mol. Biol, 43:*203-220 (2000); Finnegan, E.J., *et al., Curr. Biol., 11:*R99-R102 (2000)). Methylation of promoter regions can lead to transcriptional silencing (Metter, M.F., *et al., EMBO J., 19:*5194-5201 (2000)), but methylation in coding sequences must not (Wang, M.-B., *RNA, 7:*16-28 (2001)). DNA methylation and transcriptional silencing in mammals are well-documented processes (Kass, S.U., *et al., Trends Genet., 13:*444-449 (1997); Razin, A., *EMBO J, 17:*4905-4908 (1998)), yet they have not been linked to post-transcriptional silencing. Methylation in mammals is predominantly directed towards CpG residues. Because there is no CpG in the RL siRNA, but RL siRNA mediates specific silencing in mammalian tissue culture, it is unlikely that DNA methylation is critical for our observed silencing process. In summary, described herein, is siRNA-mediated gene silencing in mammalian cells. The use of 21 nt siRNAs holds great promise for inactivation of gene function in human tissue culture and the development of gene-specific therapeutics.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the scope of the invention encompassed by the appended claims

CLAIMS

What is claimed is:

1. Isolated RNA of from about 21 to about 23 nucleotides that mediates RNA interference of an mRNA to which it corresponds.

2. Isolated RNA of claim 1 that comprises a terminal 3' hydroxyl group.

3. Isolated RNA of claim 1 which is chemically synthesized RNA or an analog of a naturally occurring RNA.

4. An analog of isolated RNA of claim 1, wherein the analog differs from the RNA of claim 1 by the addition, deletion, substitution or alteration of one or more nucleotides.

5. Isolated RNA of from about 21 to about 23 nucleotides that inactivates a corresponding gene by transcriptional silencing.

6. A soluble extract that mediates RNA interference.

7. The soluble extract of Claim 6, wherein the extract is derived from Drosophila embryos.

8. The soluble extract of Claim 7 wherein the extract is derived from syncytial blastoderm Drosophila embryos.

9. A method of producing RNA of from about 21 to about 23 nucleotides in length comprising:
   (a) combining double-stranded RNA with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of a) under conditions in which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides in length.

10. The method of Claim 9, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

11. The method of Claim 9 further comprising isolating the RNA of from about 21 to about 23 nucleotides from the combination.

12. RNA of about 21 to about 23 nucleotides produced by the method of Claim 9.

13. A method of producing RNA of from about 21 to about 23 nucleotides in length that mediates RNA interference of mRNA of a gene to be degraded, comprising:

(a) combining double-stranded RNA that corresponds to a sequence of the gene to be degraded with a soluble extract that mediates RNA interference, thereby producing a combination; and

(b) maintaining the combination of (a) under conditions under which the double-stranded RNA is processed to RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA of the gene to be degraded, thereby producing RNA of from about 21 to about 23 nucleotides that mediates RNA interference of the mRNA.

14. The method of Claim 13, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

15. The method of Claim 13 further comprising isolating RNA of from about 21 to about 23 nucleotides from the combination.

16. Isolated RNA of from about 21 to about 23 nucleotides produced by the method of Claim 15.

17. A method of mediating RNA interference of mRNA of a gene in a cell or organism comprising:
    (a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into the cell or organism;
    (b) maintaining the cell or organism produced in (a) under conditions under which degradation of the mRNA occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

18. The method of Claim17 wherein the RNA of (a) is a chemically synthesized RNA or an analog of naturally occurring RNA.

19. The method of Claim 17, wherein the gene encodes a cellular mRNA or a viral mRNA.

20. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising:
    (a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;
    (b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of from about 21 to about 23 nucleotides, thereby producing RNA of from about 21 to about 23 nucleotides;
    (c) isolating RNA of from about 21 to about 23 nucleotides produced in (b);
    (d) introducing RNA isolated in (c) into the cell or organism; and

(e) maintaining the cell or organism produced in (d) under conditions under which degradation of mRNA of the gene occurs, thereby mediating RNA interference of the mRNA of the gene in the cell or organism.

21. The method of Claim 20, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

22. The method of Claim 20, wherein the RNA is isolated using gel electrophoresis.

23. A method of mediating RNA interference of mRNA of a gene in a cell or organism in which RNA interference occurs, comprising: (a) introducing into the cell or organism RNA of from about 21 to about 23 nucleotides that mediates RNA interference of mRNA of the gene, thereby producing a cell or organism that contains the RNA and (b) maintaining the cell or organism that contains the RNA under conditions under which RNA interference occurs, thereby mediating RNA interference of mRNA of the gene in the cell or organism.

24. The method of claim 23, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized RNA or an analog of RNA that mediates RNA interference.

25. The method of Claim 23, wherein the gene encodes a cellular mRNA or a viral mRNA.

26. A knockdown cell or organism generated by the method of claim 23.

27. The knockdown cell or organism of claim 26, wherein the cell or organism mimics a disease.

28. A method of examining the function of a gene in a cell or organism comprising:
    (a) introducing RNA of from about 21 to about 23 nucleotides that targets mRNA of the gene for degradation into the cell or organism, thereby producing a test cell or test organism;
    (b) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and
    (c) observing the phenotype of the test cell or test organism produced in (b) and, optionally, comparing the phenotype observed to that of an appropriate control cell or control organism, thereby providing information about the function of the gene.

29. The method of Claim 28 wherein the RNA introduced in (a) is chemically synthesized or an analog of RNA that mediates RNA interference.

30. A method of examining the function of a gene in a cell or organism comprising
    (a) combining double-stranded RNA that corresponds to a sequence of the gene with a soluble extract that mediates RNA interference, thereby producing a combination;
    (b) maintaining the combination produced in (a) under conditions under which the double- stranded RNA is processed to RNA of about 21 to about 23 nucleotides, whereby RNA of about 21 to about 23 nucleotides is produced;
    (c) isolating RNA of about 21 to about 23 nucleotides produced in (b);
    (d) introducing the RNA isolated in (c) into the cell or organism, thereby producing a test cell or test organism;

(e) maintaining the test cell or test organism under conditions under which degradation of mRNA of the gene occurs, thereby producing a test cell or test organism in which mRNA of the gene is degraded; and

(f) observing the phenotype of the test cell or test organism produced in (e) and, optionally, comparing the phenotype observed to that of an appropriate control, thereby providing information about the function of the gene.

31. The method of claim 30, wherein the RNA comprises a terminal 3' hydroxyl group.

32. The method of claim 30, wherein the soluble extract is derived from syncytial blastoderm Drosophila embryos.

33. The method of claim 30, wherein the RNA is isolated using gel electrophoresis.

34. A composition comprising biochemical components of a Drosophila cell that process dsRNA to RNA of about 21 to about 23 nucleotides and a suitable carrier.

35. A composition comprising biochemical components of a cell that target mRNA of a gene to be degraded by RNA of about 21 to about 23 nucleotides.

36. A method of treating a disease or condition associated with the presence of a protein in an individual comprising administering to the individual RNA of from about 21 to about 23 nucleotides that targets the mRNA of the protein for degradation.

37. The method of claim 36 wherein RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

38. A method of assessing whether an agent acts on a gene product comprising:
   (a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;
   (b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs,
   (c) introducing the agent into the cell or organism of (b); and
   (d) determining whether the agent has an effect on the cell or organism, wherein if the agent has no effect on the cell or organism then the agent acts on the gene product or on a biological pathway that involves the gene product.

39. The method of claim 38, wherein the RNA of from about 21 to about 23 nucleotides is chemically synthesized or an analog of RNA that mediates RNA interference.

40. A method of assessing whether a gene product is a suitable target for drug discovery comprising:
   (a) introducing RNA of from about 21 to about 23 nucleotides which targets the mRNA of the gene for degradation into a cell or organism;
   (b) maintaining the cell or organism of (a) under conditions in which degradation of the mRNA occurs resulting in decreased expression of the gene; and
   (c) determining the effect of the decreased expression of the gene on the cell or organism, wherein if decreased expression has an effect, then the gene product is a target for drug discovery.

41. The method of claim 40, wherein the RNA of from about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

42. A gene identified by the sequencing of endogenous 21 to 23 nucleotide RNA molecules that mediate RNA interference.

43. A pharmaceutical composition comprising RNA of from about 21 to about 23 nucleotides that mediates RNA interference and an appropriate carrier.

44. A method of producing knockdown cells, comprising introducing into cells in which a gene is to be knocked down RNA of about 21 to about 23 nt that targets the mRNA corresponding to the gene and maintaining the resulting cells under conditions under which RNAi occurs, resulting in degradation of the mRNA of the gene, thereby producing knockdown cells.

45. The method of claim 44, wherein the RNA of about 21 to about 23 nucleotides is synthetic RNA or an analog of RNA that mediates RNA interference.

46. A method of identifying target sites within mRNA that are efficiently cleaved by the RNAi process, comprising combining dsRNA corresponding to a sequence of a gene to be degraded, labeled mRNA corresponding to the gene and a soluble extract that mediates RNA interference, thereby producing a combination; maintaining the combination under conditions under which the dsRNA is degraded and identifying sites in the mRNA that are efficiently cleaved.

47. A method of identifying 21-23 nt RNAs that efficiently mediate RNAi, wherein said 21-23 nt RNAs span the target sites identified within the mRNA by the method of claim 46.

48. RNA of claim 16, isolated using gel electrophoresis.

49. RNA of claim 16, isolated using non-denaturing methods.

50. RNA of claim 16, isolated using non-denaturing column chromatography.

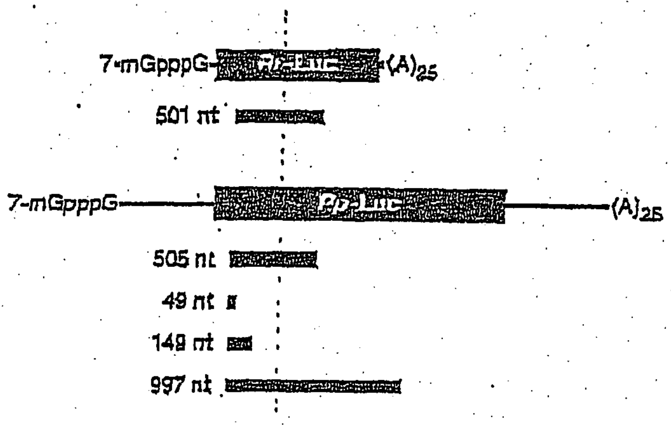


Figure 1

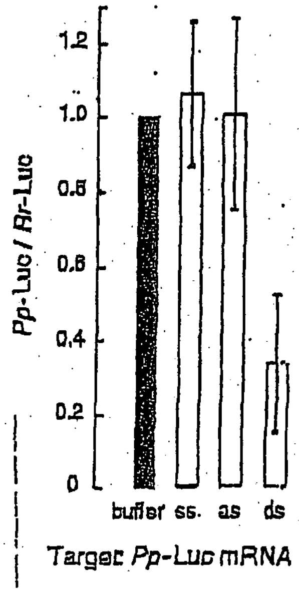


Figure 2A

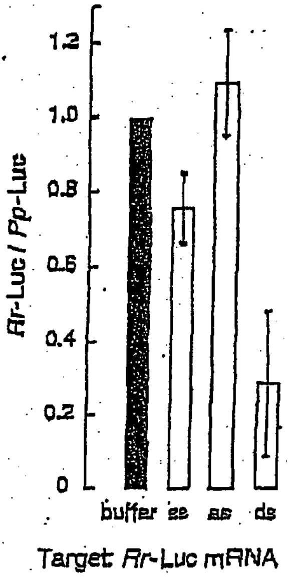


Figure 2B

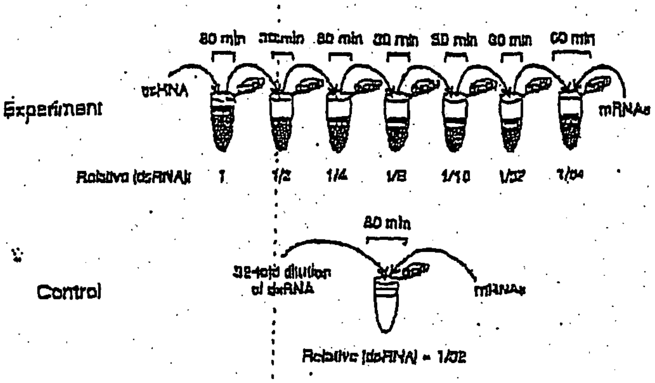


Figure 3A

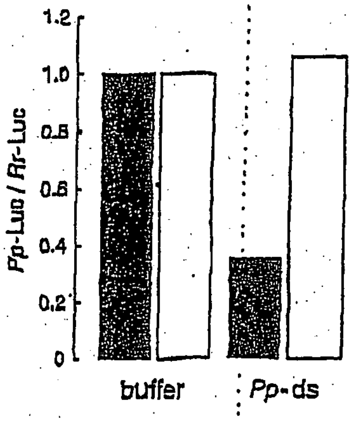


Figure 3B

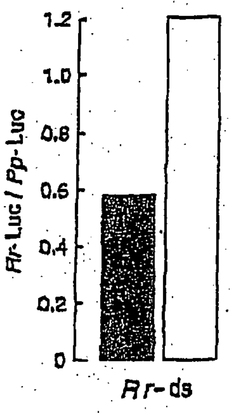


Figure 3C

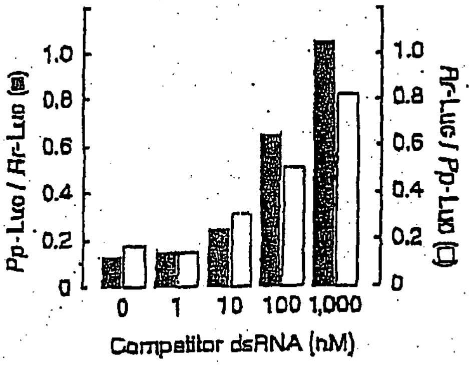


Figure 4

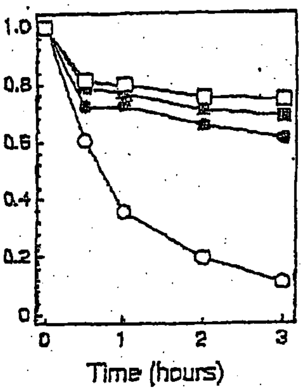


Figure. 5A

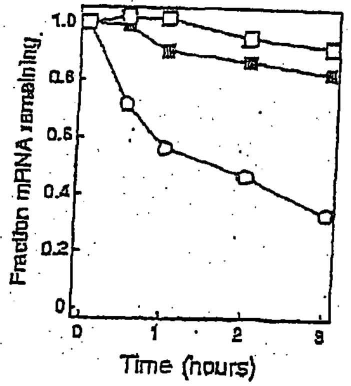


Figure 5B

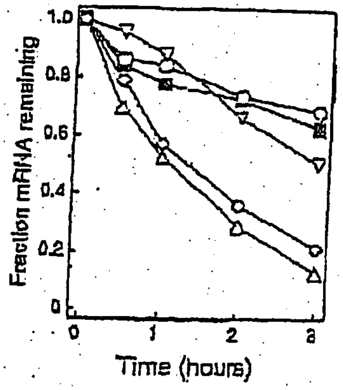


Figure 5C

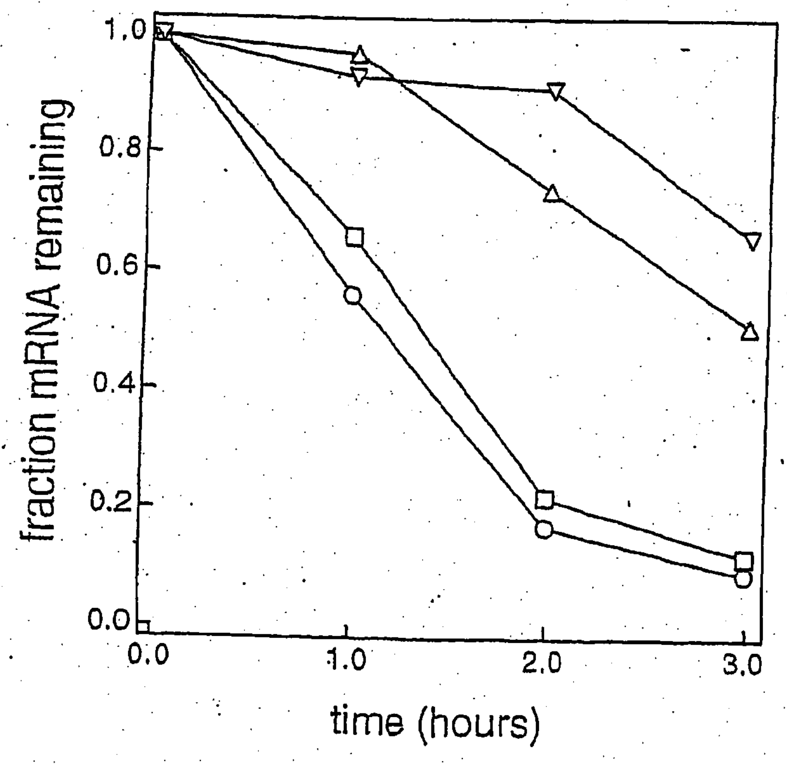


Figure 6

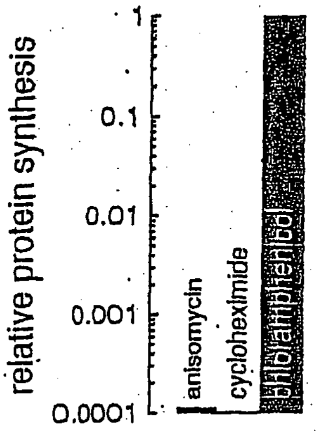


Figure 7A

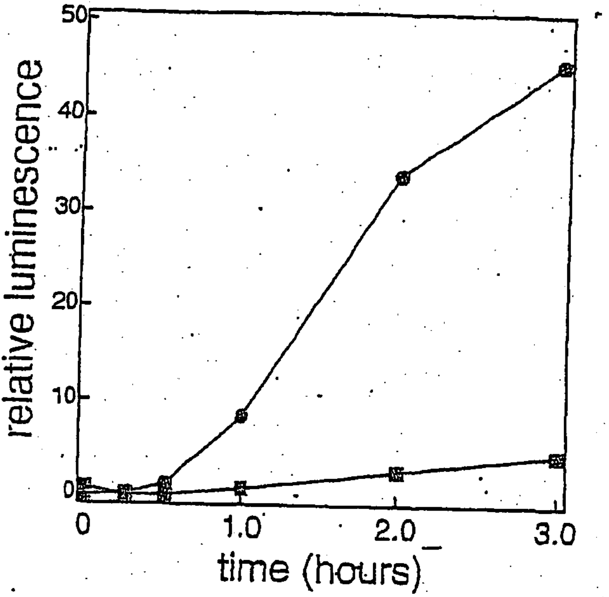


Figure 7B

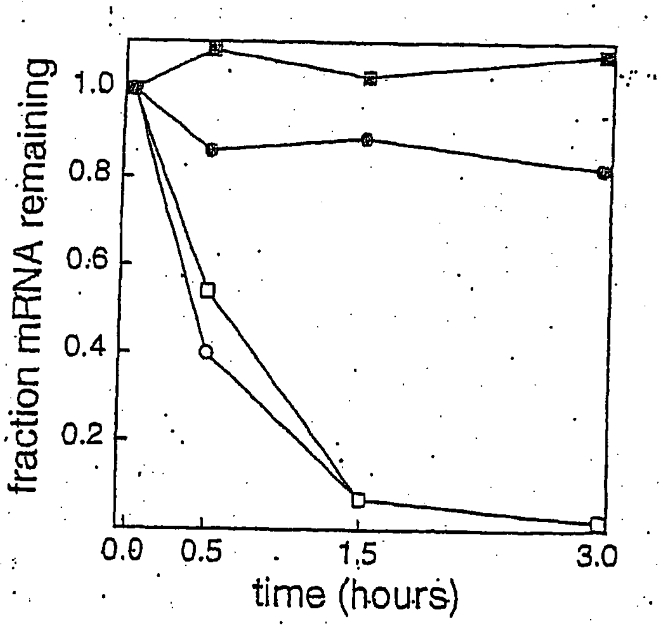


Figure 7C

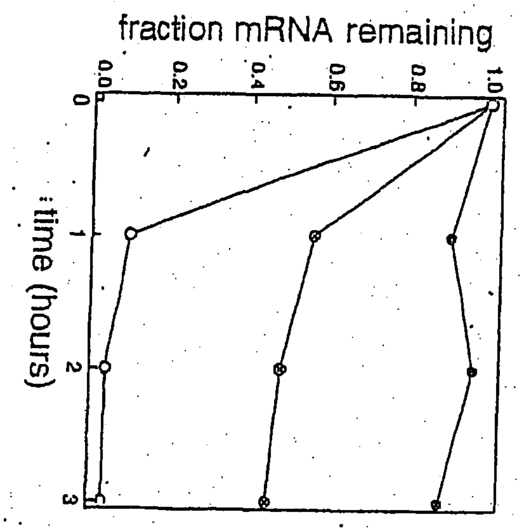


Figure 8A

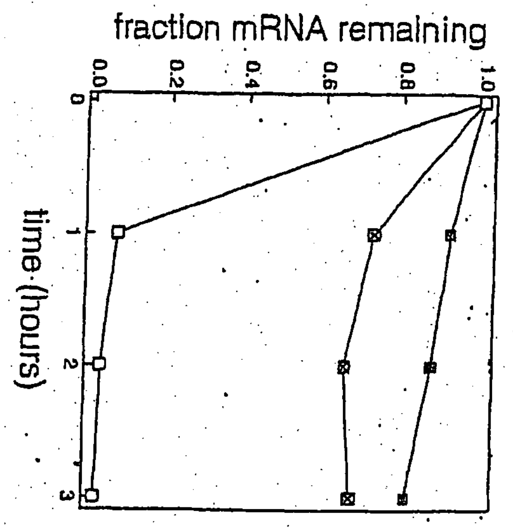


Figure 8B

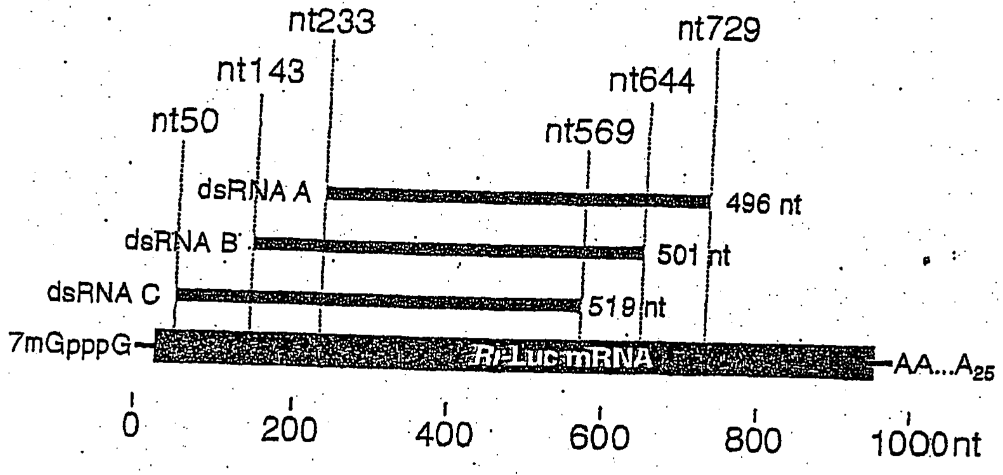
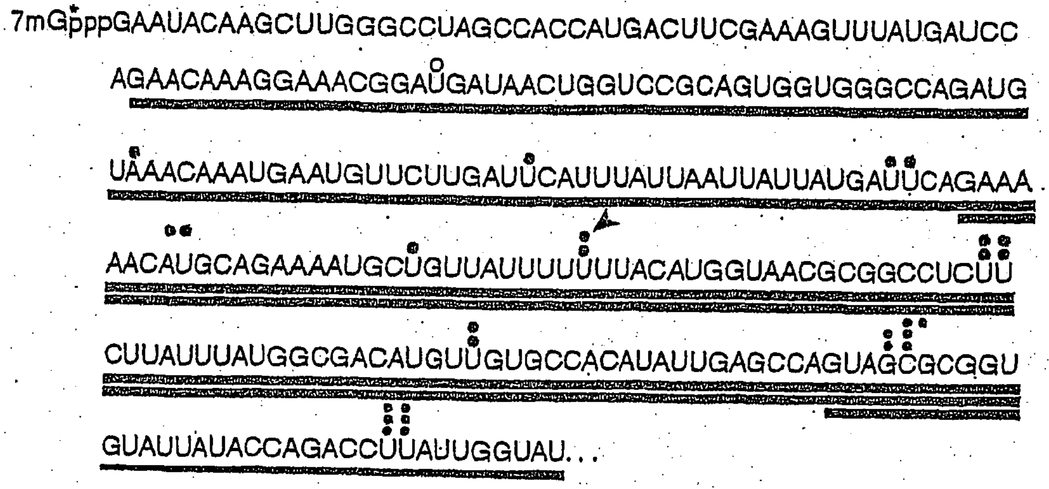


Figure 9

Figure 10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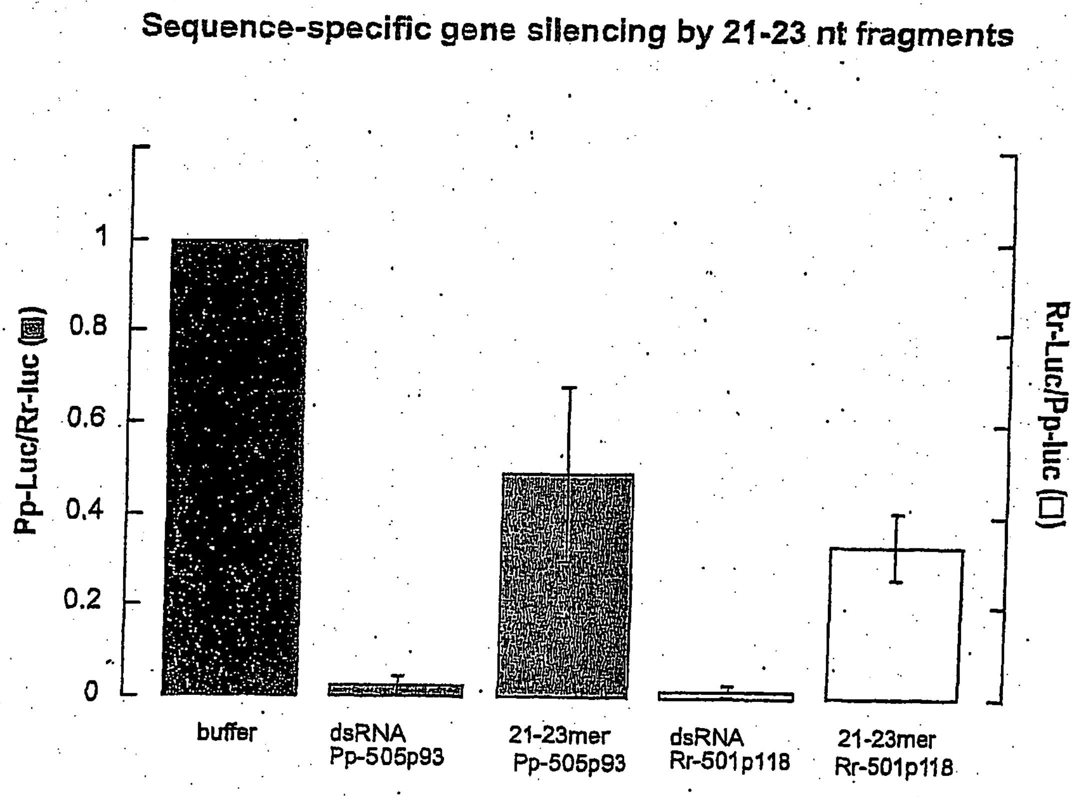


Figure 12

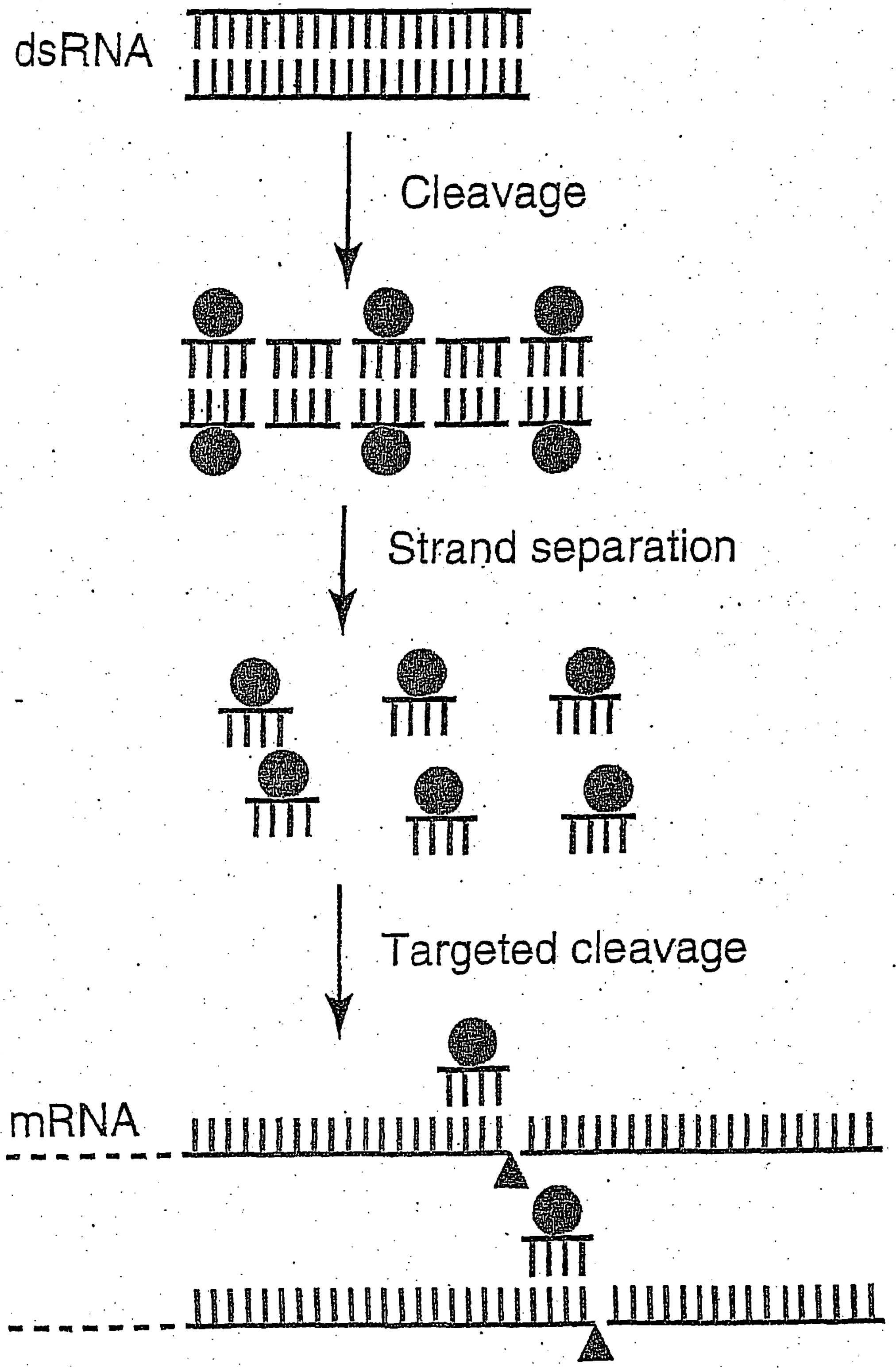
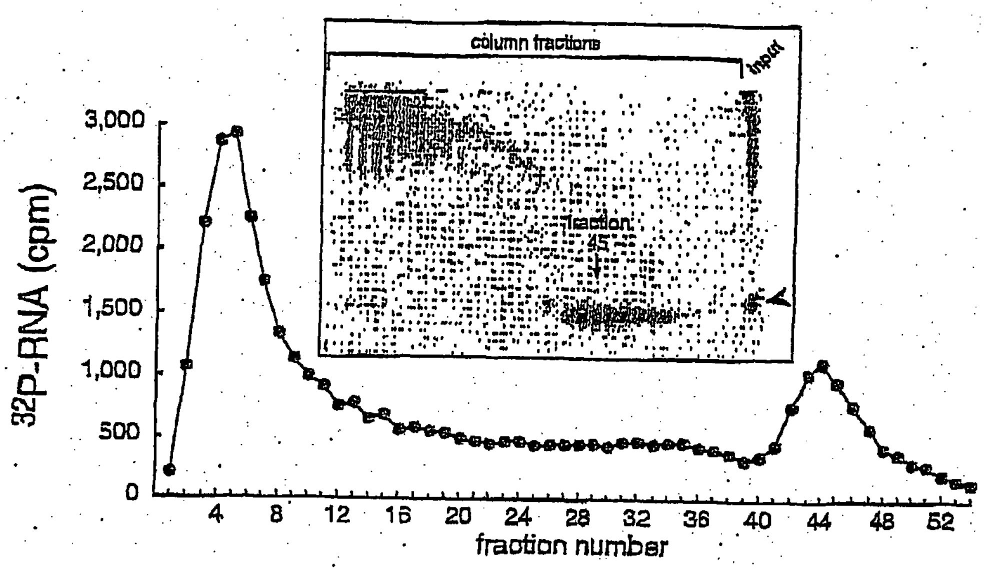


Figure 11

Figure 13A



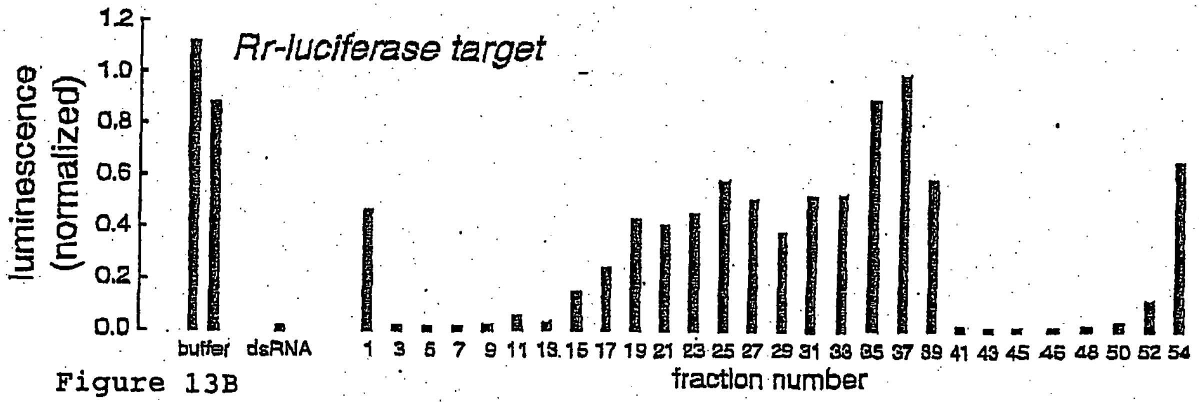


Figure 13B

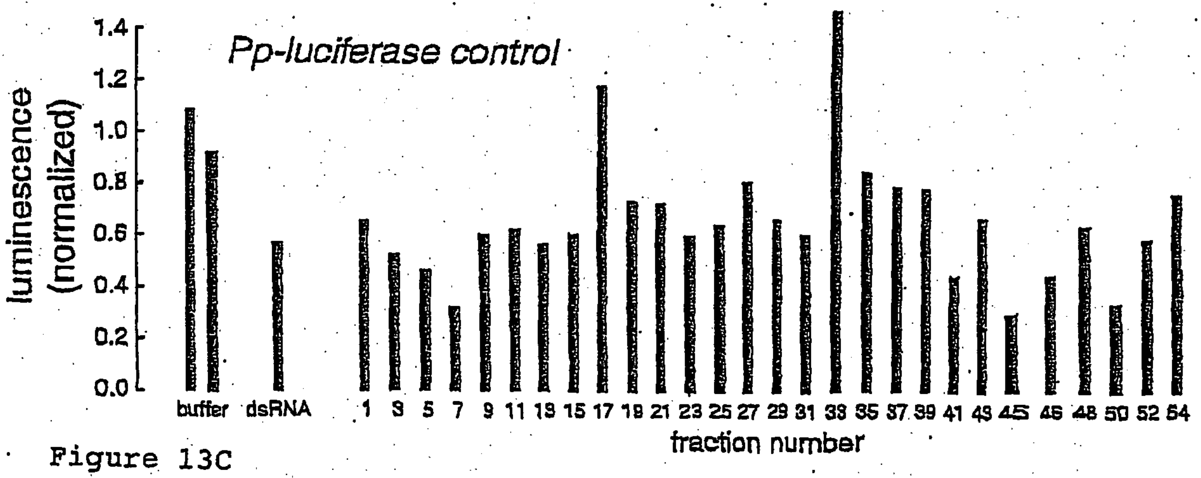


Figure 13C

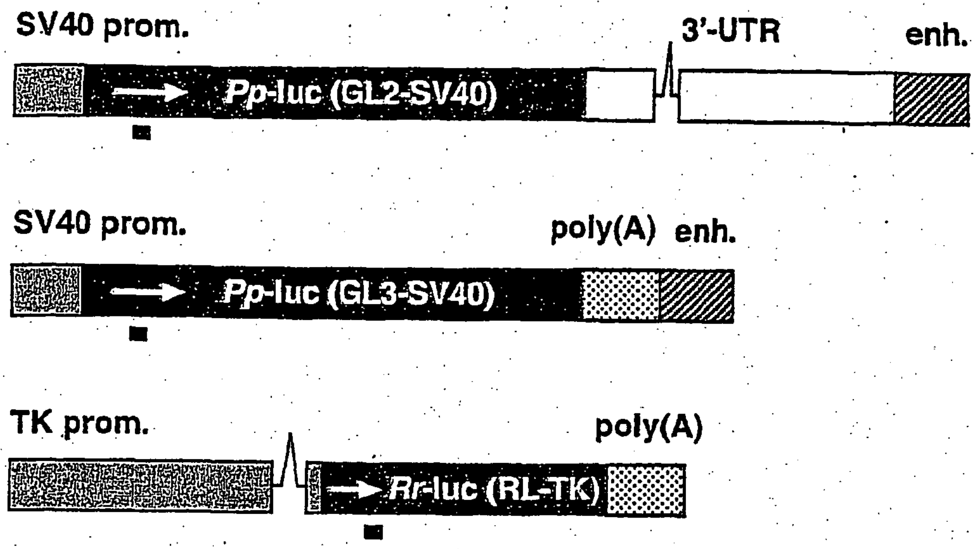


Figure 14A

siRNA duplex	
uGL2	5′ CGUACGCGGAAUACUUCGAUU UUGCAUGCGCCUUAUGAAGCU 5′
GL2	5′ CGUACGCGGAAUACUUCGATT TTGCAUGCGCCUUAUGAAGCU 5′
GL3	5′ CUUACGCUGAGUACUUCGATT TTGAAUGCGACUCAUGAAGCU 5′
invGL2	5′ AGCUUCAUAAGGCGCAUGCTT TTUCGAAGUAUUCCGCGUACG 5′
RL	5′ AAACAUGCAGAAAAUGCUGTT TTUUUGUACGUCUUUUACGAC 5′

Figure 14B

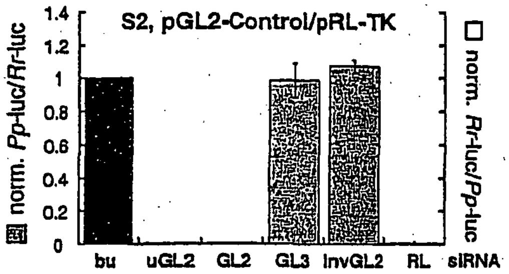


Figure 15A

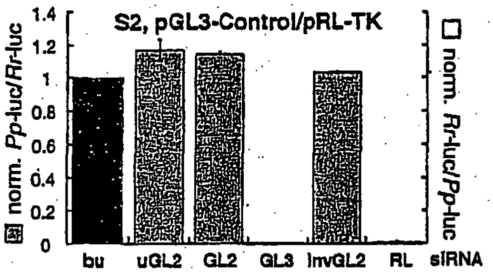


Figure 15B

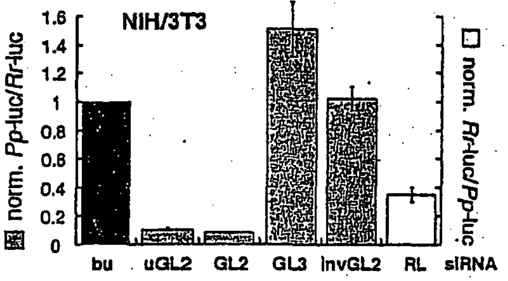


Figure 15C

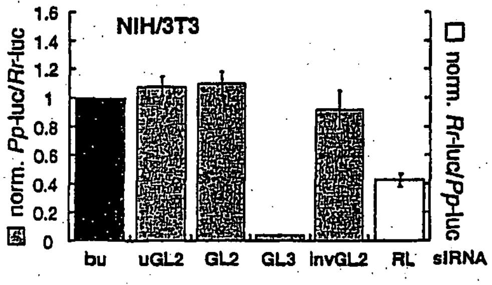


Figure 15D

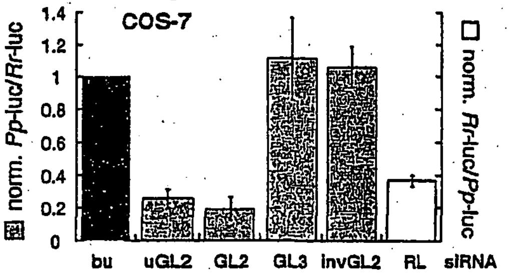


Figure 15E

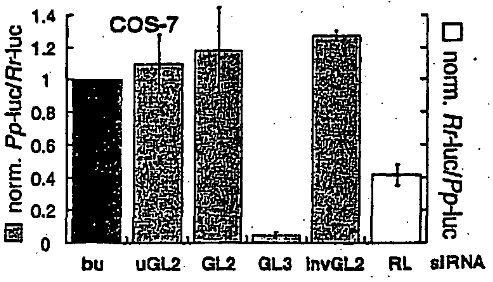


Figure 15F

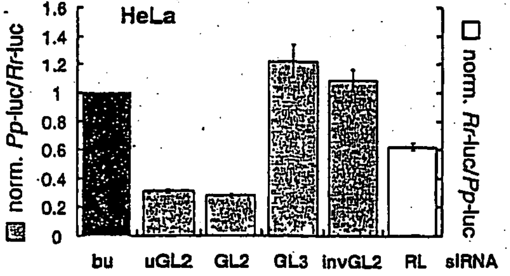


Figure 15G

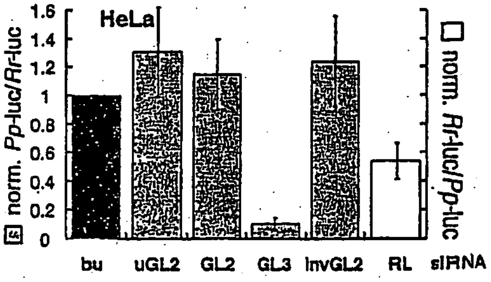


Figure 15H

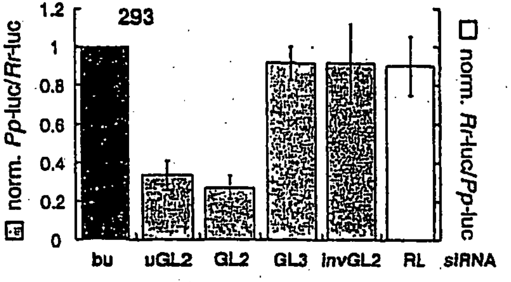


Figure 15I

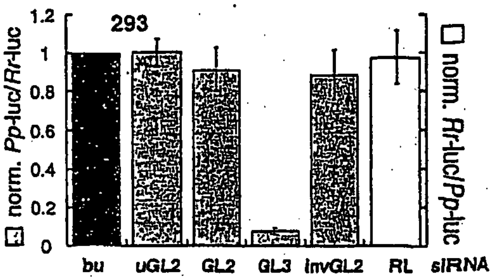


Figure 15J

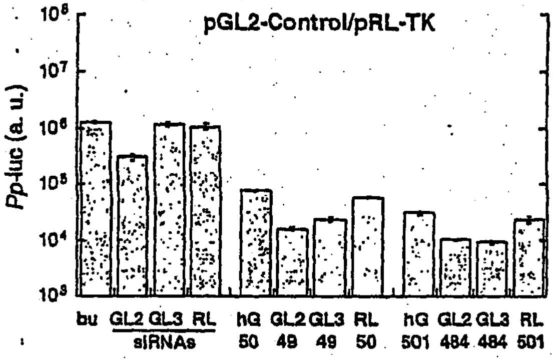


Figure 16A

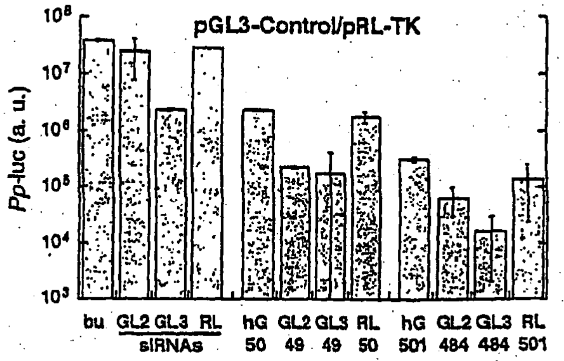


Figure 16B

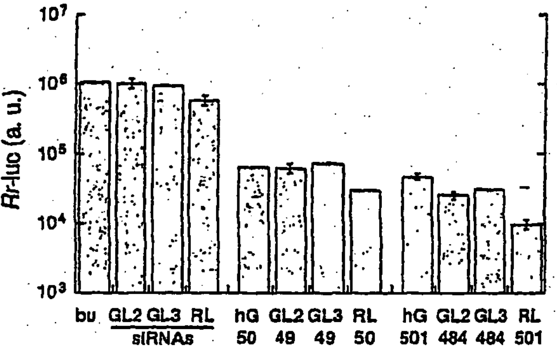


Figure 16C

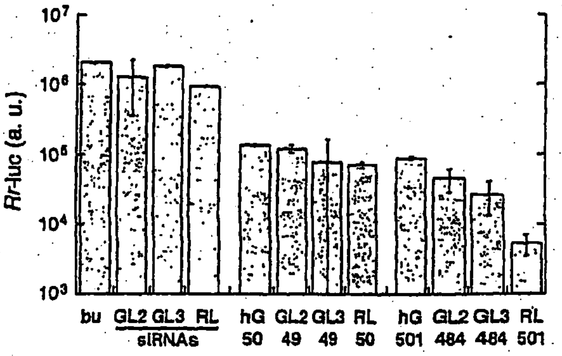


Figure 16D

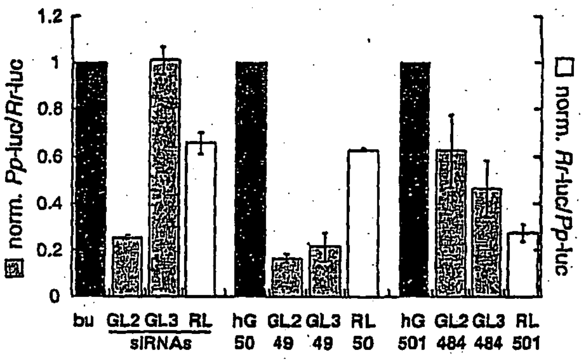


Figure 16E

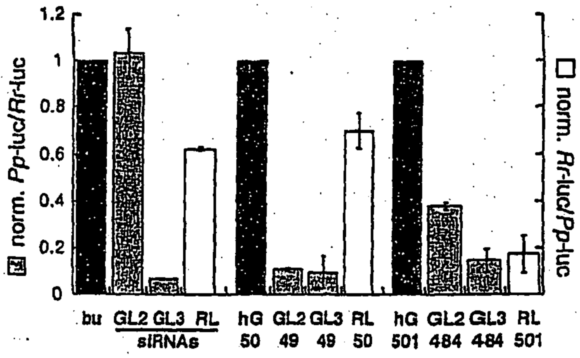


Figure 16F

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(72) Inventors; and
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(74) Agents: **COLLINS, Anne, J.** et al.; Hamilton, Brook, Smith & Reynolds, P.C., 530 Virginia Road, P.O. Box 9133, Concord, MA 01742-9133 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *with international search report*

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27 February 2003

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: RNA SEQUENCE-SPECIFIC MEDIATORS OF RNA INTERFERENCE

(57) Abstract: The present invention relates to a Drosophila in vitro system which was used to demonstrate that dsRNA is processed to RNA segments 21-23 nucleotides (nt) in length. Furthermore, when these 21-23 nt fragments are purified and added back to Drosophila extracts, they mediate RNA interference in the absence of long dsRNA. Thus, these 21-23 nt fragments are the sequence-specific mediators of RNA degradation. A molecular signal, which may be their specific length, must be present in these 21-23 nt fragments to recruit cellular factors involved in RNAi. This present invention encompasses these 21-23 nt fragments and their use for specifically inactivating gene function. The use of these fragments (or chemically synthesized oligonucleotides of the same or similar nature) enables the targeting of specific mRNAs for degradation in mammalian cells, where the use of long dsRNAs to elicit RNAi is usually not practical, presumably because of the deleterious effects of the interferon response. This specific targeting of a particular gene function is useful in functional genomic and therapeutic applications.

WO 01/075164 A3

INTERNATIONAL SEARCH REPORT

In ɔnal Application No
PCT/US 01/10188

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TUSCHL THOMAS ET AL: "Targeted mRNA degradation by double-stranded RNA in vitro" GENES AND DEVELOPMENT, COLD SPRING HARBOR LABORATORY PRESS, NEW YORK, US, vol. 13, no. 24, 15 December 1999 (1999-12-15), pages 3191-3197, XP002183118 ISSN: 0890-9369 cited in the application	6-8
Y	the whole document	9-11, 13-15, 17-25, 28-33, 38-40, 44,45

	-/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search 9 October 2002	Date of mailing of the international search report 18/10/2002
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Luzzatto, E

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HAMMOND SCOTT M ET AL: "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 404, no. 6775, 16 March 2000 (2000-03-16), pages 293-296, XP002183123 ISSN: 0028-0836	46,47
Y	the whole document ---	9-11, 13-15, 17-25, 28-33, 38-40, 44,45
A	NGÔ ET AL: "Double-stranded RNA induces mRNA degradation in Trypanosoma brucei" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 95, December 1998 (1998-12), pages 14687-14692, XP002138442 ISSN: 0027-8424 the whole document ---	9
A	HAMILTON ANDREW J ET AL: "A species of small antisense RNA in posttranscriptional gene silencing in plants" SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 286, no. 5441, 29 October 1999 (1999-10-29), pages 950-952, XP002149064 ISSN: 0036-8075 cited in the application the whole document ---	9
P,X	ZAMORE PHILLIP D ET AL: "RNAi: Double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals" CELL, CELL PRESS, CAMBRIDGE, NA, US, vol. 101, no. 1, 31 March 2000 (2000-03-31), pages 25-33, XP002208683 ISSN: 0092-8674 the whole document ---	9-11, 13-15, 17-25, 28-33, 38-40, 44-47
E	WO 01 68836 A (BEACH DAVID ;CAUDY AMY (US); HAMMOND SCOTT (US); BERNSTEIN EMILY () 20 September 2001 (2001-09-20) page 6, line 20 - line 34 page 10, line 4 -page 11, line 22 -----	9-11, 13-15, 46,47

mational application No.
PCT/US 01/10188

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsear hable (Continuation of item 1 of first heet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 1-5,12, 16,26,27,35-37,42,43,48-50 (completely), 6,9,11,13,15,20,22,30,31,3346,47 (all partially)

1)Product claims 1-5,12,16,35-37,43,48-50 relate to RNA molecules solely characterised in that they mediate RNA interference. This sole functional feature, however, is not sufficient to characterise the claimed molecules so as to allow the skilled person to clearly and unambiguosly understand the scope of the claims. Moreover, the skilled person is given no guidance, either in the claims or in the description, as to any general technical feature of the claimed RNA molecules which could allow him/her to understand, with no undue burden, which molecules fall and which do not fall within the definition of the claims.

Claims 26 and 27 relate to a knockdown cell obtained by treating it according to the method of claim 23.
However, the sole indication that a cell has been obtained by treating it with a short RNA molecule with no indication as to features of the cell, such as e.g. the kind of cell and the kind of gene which has been silenced does not allow the skilled person to clearly and unambiguously understand the scope of the claim.
Similar arguments apply to claim 42, which relates to a gene solely characterised by means of the method used for its identification.

Thus, the aforementioned claims 1-5,12,16,35-37,43,48-50, 26, 27 and 42 lack clarity (Art. 6 PCT) to such an extent as to render a meaningful search with respect to their subject-matter impossible.

2) Claims 6, 9, 13, 20, 30,46 relate broadly to a soluble extract that mediates RNA interference. However, the description only provides indications as to a Drosophila embryo extract having such a capability (see p. 22, l. 1-10, p. 28, l. 9, p. 32, l. 10, p. 36, l 14, p. 40, l. 18) and is completely silent as to any general rule that the skilled person could apply to identify, with no undue burden, other suitable extracts. The description discloses that two other lysates have been tested, namely wheat germ extract and rabbit reticulocyte lysate (see p. 28): the former showed no effect whatsoever, the latter only had a non-specific effect, not linked to RNA interference (p. 28, l. 18-20). Hence these claims lack support (Art. 6 PCT) to such an extent as to render a meaningful search of their subject-matter impossible. The search with respect to these claims has thus been limited to soluble extracts derived from Drosophila embryos.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int nal Application No
PCT/US 01/10188

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0168836	A	20-09-2001	AU	4579301 A	24-09-2001
			WO	0168836 A2	20-09-2001

Form PCT/ISA/210 (patent family annex) (July 1992)